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THE INCIDENCE OF CANCER OF THE LUNG 1932–1956

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It is clear that other factors are concerned in the phenomenal rise of the death rate from cancer of the lung in recent years apart from that of smoking, however firmly its relationship with the latter be regarded as established.

Cancer of the lung progresses rapidly from the time that it is first diagnosed, death ensuing in normal circumstances within a comparatively short time, and in consequence there has been a tendency to look for possible causes in the period immediately preceding the onset of symptoms, or their summation over a longer term. Nevertheless we have no evidence to prove that the condition itself or some precancerous state does not arise much earlier in life, lie dormant for a time and then activated perhaps by some new factor or merely as and when development extends beyond certain bounds, assume the classical character with which we are familiar.

Much of the available evidence in relation to the incubation period of cancer of various sites induced by known agents has been examined by Kennaway (1957). He was led to the conclusion that this period ranges in man from 3 to 75 years and that in consequence no specific period of general application can be established. It must, he holds, be ascertained for each site and type of cancer which arises in response to any particular carcinogenetic agent. It would seem moreover that the length of incubation in some of the instances investigated was not dependent on the intensity of action by the agent.

The investigation reported here has been pursued in a search for evidence of the time when the first changes occurred which led eventually to the manifestation of lung cancer and death from this cause. For this purpose the age-specific death rates for England and Wales from 1932 to 1956 have been analysed by cohorts and in other ways. The source of the data and the methods of calculation are set out in an appendix.

The rates which prevailed before 1932 have not been considered in detail nor in the main thesis, partly because they are less readily available, but mainly because prior to that period the death returns were probably far from complete. At that time radiological facilities were not generally available throughout the country, and the disease, being then somewhat rare, was often not thought of by the medical attendant who in consequence undoubtedly attributed many deaths from this

cause to pulmonary tuberculosis then more rife than now or to some form of pneumonia.

There may have been some improvement in diagnosis since 1932 and the suggestion has even been made that the whole of the supposed increase in lung cancer mortality has been due to this. By that time, however, the medical profession was fully alive to the existence of this entity and the consulting services of Tuberculosis Officers aided by radiography were freely used for obscure chest diseases. It is therefore submitted that further precision in the certification of this cause has not introduced any significant error.

Pulmonary cancer like that of other sites is more prone to occur in later than in earlier life and the rates in each quinquennial age group from 30 upwards have been studied. The rates below 30 suffer wide fluctuations on account of the small numbers involved as well as being subject to other errors and have for these reasons not been analysed.

Cohort analysis is not a new method for the presentation of vital statistics: it has been applied to death rates from pulmonary tuberculosis for some considerable time with success. It consists in associating deaths with the years of birth instead of with the years in which they took place. In this way the mortality suffered by a particular group or cohort of persons born at the same time can be followed through life, whereas conventional tables and graphs reflect the deaths occurring in a given year. By these means Case (1956) and others have shown that the death rate from cancer of the lung does not fall after the age of 66–70 as popularly supposed and as would appear from the graph for men of different ages who died in 1955 shown in Fig. 1, which resembles generally a series for other years published by the Registrar-General (1952) for both of the sexes. The same figure also exhibits a typical selection of cohort death rates which are seen to increase throughout their extent and not to fall in later life although the rate of increase diminishes somewhat.

The falling away of the conventional curve in later life cannot be explained by the assumption that only a proportion of the population are susceptible of whom by 65 a large number have already died, for no mathematical formula to allow for this would conform with the figures. It might, however, be inferred that the lung becomes more prone to carcinogenetic changes up to about the age of 60 or 70 and then becomes gradually less vulnerable to such changes. Nevertheless the consistent increase in the mortality rate with advancing age even if less rapid in later years as evidenced by the cohort rates would *a priori* seem to be more reasonable.

This method of analysis is particularly suited to a study of deaths which have arisen as a result of inherited characteristics or of environmental causes operating many years before death. It suffers from the disadvantage, however, that it tends to obscure the effects of influences which operated at a particular time on those of all ages such as wars, epidemics, etc.; but such events probably have little bearing on deaths from cancer.

In order to facilitate some of the arguments which follow, a hypothesis to which they lend support and which is considered worthy of further study is set out now. This is that as the intensity of the bronchocarcinogenetic agent has increased during the last half century or more, so the age of those on whom it has operated has become lower, though the age at which its effects have been exhibited possibly as a result of some other causal factor has remained unchanged. And, further, that this agent acted on both sexes with equal force but that during

incubation the resulting changes developed more actively in males than in females and that in consequence the difference between the death rates of the sexes is greater the longer the latent period.

If the age specific death rates in relation to the years of occurrence be drawn in the form of graphs a general similarity in the development of the mortality rates

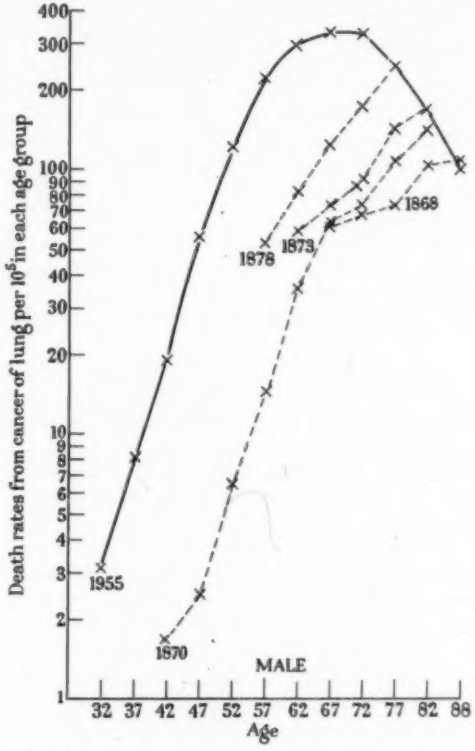

FIG. 1.—Typical age specific death rates by year of birth and by year of occurrence respectively.
Rates by year of death ————
Rates by year of birth - - - - - - -
(Rate for 1870 based on quinquennial rates.)

for the two sexes may be noted, and one is struck at once with the general uniformity of the patterns with the lines for each age group approximately parallel, the irregularities being perhaps fewer than might have been expected.

There is, however, an exception to the parallel nature of the mortality lines which is more apparent on the graph for men though it is also apparent on that for women. All the lines rise steeply at first at more or less the same angle but those representing the lower ages become nearly horizontal later, and the period

when this change in direction occurs is approximately 5 years later for each 5 years added to the age.

This apparent anomaly however, disappears if the graph for each succeeding age group is converted to represent a cohort by transposing it 5 years to the left of that for the preceding age group. This has been carried out and the result is illustrated in Fig. 2*a* and 2*b*, where the mortality of each cohort of the population as it advances in age is recorded on the same vertical line whose horizontal ordinate indicates the year of birth, on the assumption that it formed a homogeneous group born in the middle year of the five which would bring its members into the appropriate age group.

The graphs of the several age groups have now assumed a more accurately parallel course and their overlap at the higher ages has completely disappeared. Altogether the pattern has become so regularly uniform that it compels the idea that it has not arisen by chance and that there is some underlying explanation to account for it.

Also, there is now (as is to be expected from Fig. 1) no suggestion that for any cohort there is an actual decrease in the mortality suffered at any time, though the proportional increase in the mortality rate with age is clearly reduced as the lines for the older age groups are seen to be closer together than those for the younger age groups.

Apart altogether from the symmetry of the general configuration in Fig. 2*a* and 2*b* the courses followed by all the individual graph lines are remarkably consistent. These representing the age specific death rates, rise steadily in very nearly straight lines for the birth years before say 1903 and proceed more or less horizontally for those born after that year. This indicates that in the earlier period the death rates at a given age increased by a constant ratio as the years of birth succeeded each other.

It seems difficult to account for the regular proportional increase of the death rates either on the assumption of changes in inherited characters or of alterations in the environment. Any such changes would almost certainly have taken place much more irregularly and it would be very unlikely for them to have increased in geometrical rather than in arithmetical progression. On the other hand if the increase were due to a latent malign process having operated for a longer period it would not be surprising if for each additional year of duration of the malign process the increase had been in a constant ratio.

This alternative explanation is to the effect that the disease process had lasted an equal time longer in those born in any year than in those born in the preceding year, the regularity of the increase in the death rates being then dependent on a constant rate of development. This supposition may be considered inherently reasonable: it implies the start of the process at progressively younger ages among the cohorts under review.

It might be argued that this would still take no account of the regularity of the lowering of the age attacked over such a long period. But if the age were lowered by one year for each advance in the year of birth by one year, then the actual start of the process in all the groups would have been simultaneous and no question of regularity would arise. And even if the age were lowered by a period although not a whole year yet only a little short of it, the process would then have begun in a very few years over the whole range of the persons involved and the regularity should not cause surprise.

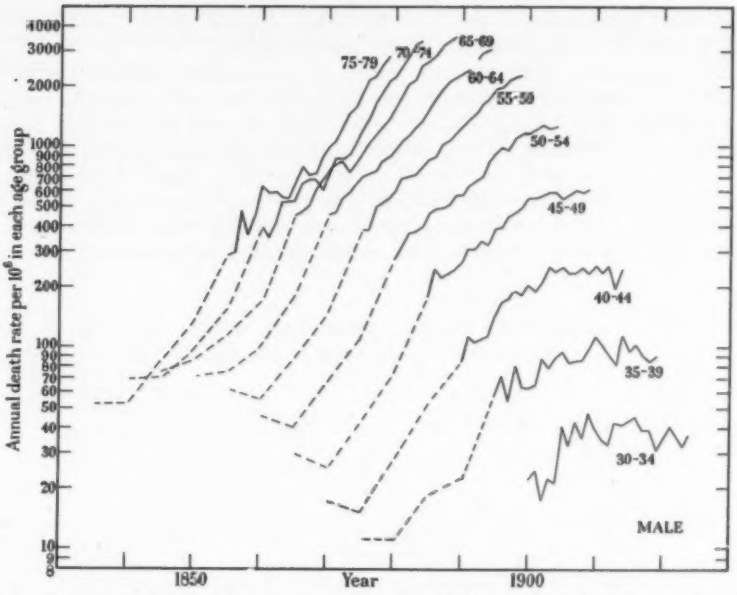

FIG. 2a.—Age specific death rates by year of birth. (Dotted lines derived from quinquennial rates prior to 1932.)

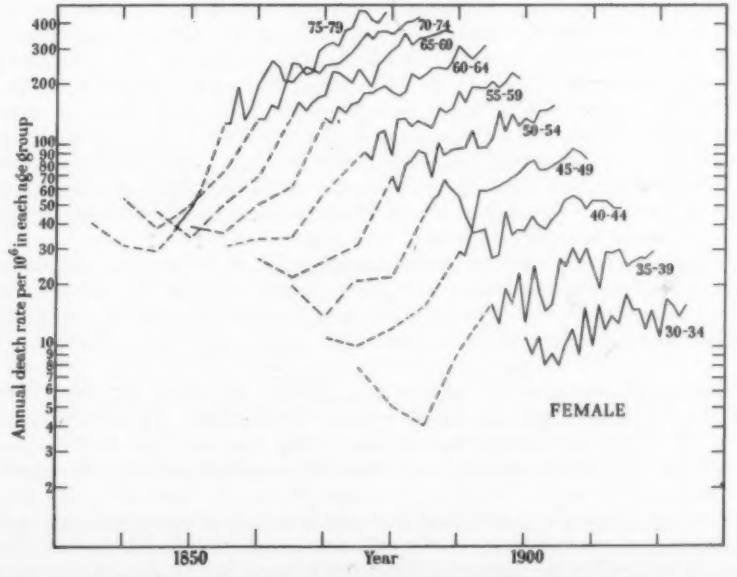

FIG. 2b.—Age specific death rates by year of birth. (Dotted lines derived from quinquennial rates prior to 1932.)

That this explanation of the individual age specific death rates is veridical is the first part of the hypothesis already put forward.

If the relationship between the graphs of the various age groups be again examined it becomes clear from their general parallel character that the proportion which the death rate in a given age period bore to that in the next lower age period was nearly constant. These proportions have been calculated in every case shown in the figures, and their mean value is given in Table I for each of the sexes. This table also gives the relation between these proportions for each step in advancing age : in this way the proportional increase in the difference between the death rates for the two sexes with rising age may be measured.

TABLE I.—*Mean Proportions Between Mortalities at One Age Period and that Next Below for Cohorts Born in the Same Year*

Age period	Males	Females	Ratio of mean male to mean female proportion
80–84	1·06	1·09	0·97
75–79	1·34	1·26	0·07
70–74	1·28	1·35	0·95
65–69	1·56	1·47	1·06
60–64	1·73	1·70	1·02
55–59	2·07	1·61	1·29
50–54	2·26	1·86	1·215
45–49	2·62	1·76	1·49
40–44	2·67	2·07	1·29
35–39	2·63	2·03	1·30

If the former proportions subsisted before the records here studied commenced, then by applying them to the known rates among the older groups born in any one year it would be easy to calculate the rates that existed in the younger groups born in the same year. There is no evidence of the death rates below the age of 30, but if the assumption be made that the proportional increases at ages below 30 were approximately the same as those in the thirties then the notional mortality rates at these lower ages could similarly be calculated. And also by applying the ratios between the proportional increases for males and females respectively, the age can be readily discovered at which these notional mortality rates would have been the same for both sexes for each birth year.

The mean values of the age of this sex equality of the notional death rates for each birth year calculated from every pair of death rates for the two sexes already given are shown in Fig. 3. The ages so calculated are remarkably consistent and the mean values are derived from ages which do not vary by more than a year or two.

A reasonable explanation of the sex differences would be that both were attacked at or about these ages by the same noxious influence but that the development of the resulting changes was greater in the one sex than in the other. The veridical nature of this explanation forms the second part of the adumbrated hypothesis.

The time when the individual cohorts reached a particular age and were attacked, as we now assume, can be related to calendar years and in Fig. 4 the ages of those assailed are plotted against the years in which the assault took place.

The age scale has been reversed in order that the increasing intensity of the bronchocarcinogenetic force acting at progressively lower ages can be more conveniently illustrated.

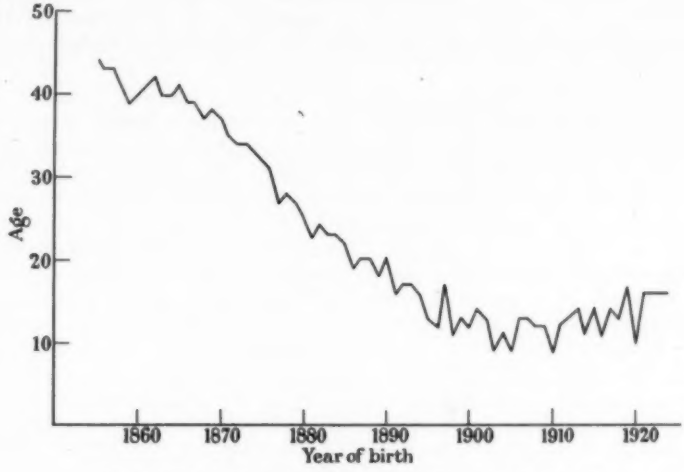

FIG. 3.—Age of sex equality of mortality rates (notional) for cohorts by year of birth.

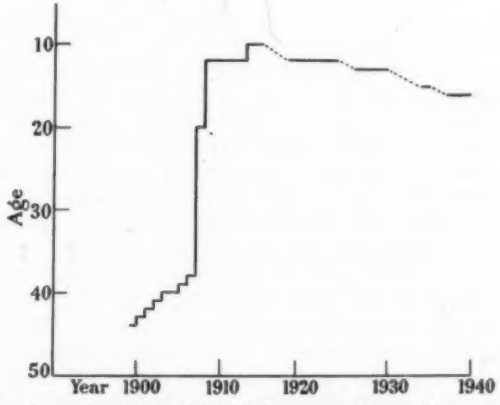

FIG. 4.—Age of hypothetical initial damage and year of its occurrence.

This diagram is therefore submitted as a representation of the increase in the intensity of at least one of the causes of cancer of the lung from 1900 to 1940. It reflects an explosive eruption of this force somewhere about 1905–10, but also gives some hope for the future in that there has been some regression since 1915, though the full force of the maximum in that year may not yet have been felt.

DISCUSSION

This conception of the development of lung cancer is not in conflict with any evidence that the incidence of the disease is higher in one section of the community than in another provided always that the proportion of the more susceptible section to the whole population has not changed materially over the period studied.

Although urbanization has progressed to a very marked degree in the last 50–100 years, nevertheless there has been a continued relief of overcrowding in the centres of large towns and an improvement in the environment with a corresponding migration to areas of lower population densities in the periphery. The net result may well be that the proportion of those at greater risk on account of living in towns has remained unchanged.

It may be rather more difficult to reconcile the acknowledged association of smoking and lung cancer with the theory, but there are possible ways in which this can be done. It is, on the face of it, unlikely that changes in smoking habits should have affected boys let alone girls so young, even if they concerned the character of the tobacco rather than the amount smoked. It is therefore most improbable that smoking was an aetiological factor in the initial stages of cancer if it does commence as early as here envisaged. On the other hand it would not be entirely unreasonable to suppose that bronchial or pulmonary changes produced by other factors themselves encourage a desire to smoke. However, it may be that smoking habits have not really altered sufficiently to effect to a significant extent the proportion of smokers in the community.

It should be made abundantly clear that the hypothesis advanced represents only one possible explanation of the data examined being little more than a conjecture, and that if the death rates prior to 1932 are studied the resulting picture of events becomes equivocal.

It may be that these rates, based as they are on most incomplete and faulty diagnosis, bore little relation to the true incidence of the disease; and moveover that during the period when greater accuracy was being established, improvement in the certification in all the age groups did not occur at the same time. In consequence the ratios between the rates would have no kind of significance. The writer is inclined to this view, but they cannot nevertheless be entirely ignored.

The ratios between the rates that prevailed in each age group and in that next preceding it, derived from the quinquennial death rates from 1907 onwards, are shown in Table II. Stocks (1953 and private communication) has pointed out that those ratios which are determined by the death rates from 1926 to 1934 constitute a very marked maximum in every cohort. It is rather difficult to account for this, but he considers that it is consequent upon a sudden diagnostic improvement in those years due to the publications regarding lung cancer which appeared at that time.

On the other hand if faulty diagnosis influenced the rates in all the age groups similarly then the ratios under consideration would be unaffected. On this assumption the notional age of sex equality has been calculated using the averages of the ratios in the table, which do not differ significantly from those already given. The ages found in this way fell gradually from 50 for the cohort born in 1830 to 40 for that born in 1860, which would imply, if the tentative hypothesis be accepted, that these cohorts were acted upon carcinogenetically between 1880

TABLE II.—*Proportions Between Mortalities at One Age Period and that Next Below for Cohorts Born in and around the Same Year.*

(Based on quinquennial rates)

Age period	Birth years of cohorts															
	1835	1840	1845	1850	1855	1860	1865	1870	1875	1880	1885	1890	1895	1900	1905	1910
Males																
40–44	—	—	—	—	—	—	—	—	1·36	2·45	2·89	3·95	2·76	2·81	2·92	2·65
45–49	—	—	—	—	—	—	—	1·47	2·94	2·82	3·58	3·15	2·58	2·84	2·47	—
50–54	—	—	—	—	—	—	1·33	2·64	2·54	3·37	2·32	2·18	2·48	2·26	—	—
55–59	—	—	—	—	—	1·19	2·18	2·24	3·10	2·28	2·05	2·26	2·11	—	—	—
60–64	—	—	—	—	1·25	1·89	2·08	2·46	1·86	1·74	1·94	1·90	—	—	—	—
65–69	—	—	—	1·23	1·49	1·67	1·96	1·75	1·50	1·73	1·71	—	—	—	—	—
70–74	—	—	0·94	1·13	1·14	2·06	1·51	1·18	1·44	1·49	—	—	—	—	—	—
75–79	—	0·76	1·21	1·37	1·75	1·33	1·18	1·35	1·48	—	—	—	—	—	—	—
80–84	0·79	0·96	1·09	1·42	1·17	0·83	1·20	1·41	—	—	—	—	—	—	—	—
Females																
40–44	—	—	—	—	—	—	—	—	1·25	2·40	4·0	2·78	2·46	2·25	2·18	2·32
45–49	—	—	—	—	—	—	—	1·27	2·10	1·83	2·56	1·96	1·78	2·30	1·81	—
50–54	—	—	—	—	—	—	1·10	1·86	1·53	2·50	1·90	1·90	2·05	1·88	—	—
55–59	—	—	—	—	—	1·26	1·56	2·23	2·44	1·95	1·59	1·82	1·74	—	—	—
60–64	—	—	—	—	1·16	1·47	1·80	2·04	1·96	1·59	1·79	1·68	—	—	—	—
65–69	—	—	—	0·87	1·39	1·38	2·16	1·52	1·31	1·78	1·57	—	—	—	—	—
70–74	—	—	0·83	1·47	1·48	1·70	1·46	1·26	1·57	1·28	—	—	—	—	—	—
75–79	—	0·54	1·03	1·46	1·64	1·56	1·07	1·37	1·35	—	—	—	—	—	—	—
80–84	0·78	1·00	1·26	1·30	1·26	0·94	1·37	1·27	—	—	—	—	—	—	—	—

and 1900 at a progressively lower age. The average annual rates derived from the quinquennial rates prior to 1932 which have been used in these calculations are shown as dotted lines in Fig. 2*a* and 2*b*.

Arraigned against the theory also is the evidence that even before this century cancer of the lung was well known in younger as well as in older subjects. Nevertheless, exact diagnosis at that time was only possible for the very few cases for which autopsies were conducted and the number of these was too small for statistical analysis. Also, there may have been some particular hazard to account for some of the recorded examples such as was the case among the Joachimsthal miners.

A fallacy too has been introduced into the analysis by the inclusion of deaths due to secondary deposits in the lung among those due to primary growths. With the known increase of the latter their proportion of the total might be thought to have increased also. But if local changes in the tissues predisposed the lungs to primary carcinoma they may also have made them a more favoured seat for secondary involvement, the proportion of the two varieties of neoplastic changes remaining nearly constant.

On the other hand the theory finds some support from the New Zealand experience (Eastcott, 1957) where those who emigrated from Great Britain in middle life were found to die more frequently from lung cancer after many years in that country than those who emigrated at a much younger age. It is interesting also to note that Scott (1956) has found that there is a slightly significant correlation in certain London Boroughs between the standardized mortality ratio for lung cancer and the percentage of residents born in London.

It may also be noted that the conception of a more intense carcinogenetic agent acting at a younger age rather than affecting the virulence of the disease

would seem to accord with Kennaway's (1957) findings, and is certainly not contrary to his views already expressed.

SUMMARY AND CONCLUSIONS

It is submitted that sufficient evidence has been adduced of the initial development of cancer of the lung or of some predisposing condition many years before it becomes manifest to warrant further statistical and epidemiological study.

The findings here reported indicate that the development of cancer of the lung now seen may have been at least partly determined during the 'teen ages. Epidemiological investigations of cases coming to our knowledge should therefore include that period of earlier life.

The suggestion is made that one explanation of available data is that early in the century there was an almost explosive increase in the bronchocarcinogenetic forces. It is not the purpose of this report to discuss the nature of these forces which might have operated or their origin, but thoughts turn involuntarily towards the rapid development at that time of motor-car traffic. Studies should, however, look for other changes in the environment which may have occurred then, including those of the intensity of radiations to which the population was exposed.

The acceptance of the hypothesis put forward and the arguments propounded do not imply that more than one aetiological factor is not concerned in the final efflorescence of the disease provided forces other than the one here resolved have been directed to a constant proportion of the population during the period studied. Such other factors may be a requisite before the influence determining the invasion considered here could operate and be later precipitating causes essential to the final outcome.

Statistical analyses along similar lines to those here presented should be made of the available data in other parts of the world and if possible in particular groups of individuals or in communities in this country.

APPENDIX

1. All the death rates have been calculated from the actual deaths and estimates of the population as recorded in the several age groups by the Registrar-General of England and Wales in Part III of his statistical reviews for the years 1932–56.

2.1. From 1932 to 1939 the deaths from cancer of the lung have been taken as those from cancer and other malignant diseases of the respiratory organs [47] less an estimate of the number of deaths from cancer of the larynx and trachea based on the trend of deaths from these causes [47(*a*)] from 1940 to 1949.

2.2. From 1940 to 1949 the deaths from cancer of the lung have been taken as those from cancer and other malignant tumours of the lung and pleura [47(*b*)].

2.3. From 1950 to 1956 the deaths from cancer of the lung have been taken as those from malignant neoplasms of the trachea and bronchus and lung specified as primary, together with those of malignant neoplasm of lung and bronchus unspecified as to whether primary or secondary. No deductions have been made to allow for deaths from cancer of the trachea as the number of these was considered to have probably been insignificant.

2.4. The figures for non-civilian deaths which are recorded separately for certain years have been added where appropriate.

3. The populations have been taken from the estimates for the Total population except for the years 1940–42 when they were not published. For these years the populations have been taken by assessing the proportional changes which appeared between the estimates for 1939 and 1943 for those under 65, and by accepting the estimates for the Home population for those above 65.

4. All quinquennial rates are taken from ' Studies on Medical and Population Subjects ', No. 13 issued from the General Register Office.

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ADDENDUM

Since the submission of the manuscript of this article the Statistical Report of the Registrar-General for 1957 has been published. The age specific death rates from cancer of the lung, derived from the data now published, conform closely to the patterns described for earlier years.

THE INCREASE IN THE MORTALITY DUE TO CANCER OF THE LUNG IN THE LIGHT OF THE DISTRIBUTION OF THE DISEASE AMONG THE DIFFERENT SOCIAL CLASSES AND OCCUPATIONS

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I. *Changes in Diagnosis as a Possible Cause for the Increase in Mortality Due to Cancer of the Lung*

In a paper by Greenwood (1948), there occurs a sentence which may fitly be regarded as an axiom of medical statistics :

> " One should never believe that a disease is becoming more or less deadly until all other explanations have been excluded."

Obviously, among such " other explanations " is a change in diagnosis, and he quotes the following example.

> " Change of fashion in certification due to increasing knowledge may affect comparability. For example, between 1861–70 and 1901–10 the rate of mortality from Acute and Chronic Nephritis (inflammation of the kidneys) increased ; the index number (usually called age standardized rate of mortality) for males increased from 153 to 435. In 1911–20 it fell to 406, and in 1921–30 to 303. It would be rash to infer that the ' disease ' increased and then diminished. A much more probable explanation is that after 1870 doctors increasingly spoke of nephritis, and eschewed certifying a mere physical sign—dropsy—which might be due to many diseases ; and after 1910 they preferred to certify arteriosclerosis, thinking, no doubt rightly, that the change in the kidneys was secondary to changes in the vascular system."

Paraphrasing Polya's dictum about " chance as the ever-present rival conjecture " (Polya, 1954), we may, in medical statistics, speak of a change in diagnosis as the ever-present rival conjecture which ought to come to our mind when examining striking changes in the incidence of non-epidemic disease, and shall accordingly in this paper consider, in Section I, the possibility that the spectacular rise of the death rate due to cancer of the lung (C.L.) may be partly due to a change in diagnosis.

The social class gradient for cancer of the lung

The hypothesis of a change in diagnostic habits finds support in the emergence of a definite social class gradient according to the information made recently available by the Registrar-General (Registrar-General, 1958).

Up to the latest publication by the Registrar-General referred to above, hardly any connection has been noticed between social class and the incidence of lung cancer, except in Copenhagen (Clemmesen and Nielsen, 1951) on the

basis of which the following forecast was made in 1952 by Stocks " that there is little doubt that the census analysis in this country will eventually reveal the same thing ". The data made available on this matter by the Registrar-General (1938) in his last but one volume on Occupational Mortality did not suggest any well defined association. Now, twenty years later, there appears a definite social class gradient for men aged 20–64 (the range of age classes for which the Standardized Mortality Ratio is calculated).

The Standardized Mortality Ratio may be briefly explained thus. The Registrar-General calculates " standard deaths " which are the numbers expected on the hypothesis that the age specific death rates for a given occupational group are the same as for the general population. They are calculated by applying the general mortality rates of Table 3 of the Registrar-General's (1938) Decennial Supplement, for all males, all married women, or all single women, as the case may be, at the appropriate age-groups, 20–25–35–45–55–65, to the census population of the occupational group and summing the products. Thus they represent the deaths which would result in an occupation group if that group were exposed at each age to the standard mortality risks. The S.M.R. is the percentage ratio of the deaths actually registered for the group, to the calculated standard deaths.

The table published by the Registrar-General (1958) on page 36 of the publication referred to above clearly shows the change in social class pattern for cancer of the lung in males between 1920 and 1950 (Table I).

TABLE I.—*Cancer of Lung and Bronchus: S.M.R's* (20–64), 1949–53 *Compared with Previous Analyses*

	Males			Married women	
	1921–23	1930–32	1949–53	1930–32	1949–53
Social class I	100	107	81	95	119
II	109	95	82	100	95
III	97	100	107	108	102
IV	79	92	91	81	98
V	124	114	118	94	96

On the other hand, a pronounced social class gradient was always a feature of the Standardized Mortality Ratio due to pneumonia, bronchitis and bronchiectasis, and also influenza for which, however, there was no gradient in 1930 for married women and only a very slight one for men (Table II).

The emergence of a social class gradient for a disease which so far did not show any such a gradient is a rather unusual feature, which may be most naturally accounted for by a change in diagnostic habits. Let us suppose that there is a class of borderline cases which, before 1930, were diagnosed as bronchitis, but which nowadays would rather be diagnosed as the malignant disease. If the cause of death was actually bronchitis, the statistics would show a social class gradient and if the cause of death for the same type of borderline cases is now rather given as the malignant disease, this would cause a social class gradient to appear in the Standardized Mortality Ratio for the malignant disease.

The inference of a change in diagnosis is valid no matter what assumption we make about the social gradient, whether it is apparent only, or real.

(*a*) If mortality due to C.L. is *not* intrinsically differentiated according to social class and occupation, the absence of a social class gradient for that disease in

TABLE II.—*Influenza, Pneumonia and Bronchitis: S.M.R's (20–64) by Social Class, 1949–53 Compared with Previous Analyses*

	Social class	Males		Married women	
		1930–32	1949–53	1930–32	1949–53
Influenza	I	95	58	102	64
	II	101	70	101	70
	III	94	97	97	105
	IV	107	102	100	113
	V	105	139	104	116
Pneumonia	I	71	53	72	61
	II	80	64	77	73
	III	91	92	96	96
	IV	109	105	105	113
	V	139	150	133	132
Bronchitis	I	31	34	27	35
	II	57	53	56	49
	III	91	98	99	101
	IV	124	101	119	123
	V	156	171	155	154

1930 and its emergence in 1950 would justify the conclusion that what is now diagnosed as C.L. was, in 1930, diagnosed as a disease with social class gradient, such as bronchitis, respiratory tuberculosis, etc., and is thus today wrongly diagnosed as C.L.

(*b*) If mortality due to C.L. *is* intrinsically different according to social class and occupation, the emergence of a social class gradient in 1950 would mean that what is now diagnosed as C.L. is correctly so diagnosed, but was incorrectly diagnosed in 1930 as chronic bronchitis, respiratory tuberculosis, pneumonia, etc. In collections of pathological specimens there is no lack of evidence for cases of C.L. that have been wrongly diagnosed as respiratory tuberculosis.

There is a high probability that around 1930, before the introduction of the National Health Service, diagnosis of lung cancer, depending as it did on special techniques, was more likely to be made in patients of Social Class I than in those of Social Class V. This would account for both, the overall increase in mortality due to C.L. and the special increase in Social Class V between 1930 and 1950.

Whatever assumption we make about the social class gradient in C.L., the sudden increase in mortality due to nominal C.L. is explained, to some extent at least, namely as far as social class gradient exists, by a change in diagnosis.

The conceivable objection to that conclusion is that the emergence of the social class gradient was due to the greater numbers in 1950 compared with what they were before. Considering, however, that in 1930 the mortality within a social class must have had at least as many cases as there are now within some occupational groups, occupational mortality ought to have shown these differences at least between the 5 classes, which was not the case.

II. *The Excess Male Mortality for Cancer of the Lung*

There is, however, a third possibility, and most likely all these " possibilities " are not mutually exclusive but can co-exist in certain proportions. The rise in mortality due to C.L. may be real, not only apparent, and the greater number of cases may have brought to light the social class gradient always inherent in

C.L. But the very fact of a social class gradient points again to an explanation which is decidedly different from that given by Doll and Bradford Hill (1952).

Cancer of the lung belongs to the class of diseases with a pronounced excess male mortality ratio.

TABLE III.—*Excess Male Mortality Ratio for C.L.*

1937	.	1·02
1938	.	1·00
1939	.	0·96
1940	.	1·02
1941	.	1·08
1942	.	1·07
1943	.	1·16
1944	.	1·19
1945	.	1·18
1946	.	1·25
1947	.	1·26
1948	.	1·29
1949	.	1·34
1950	.	1·39
1951	.	1·48
1952	.	1·53
1953	.	1·52
1954	.	1·65
1955	.	1·67
1956	.	1·71

An exception to the rule of excess male mortality for C.L. amongst a selected group of people is discussed below (p. 503).

Excess male mortality in man

We have arrived to-day at the conclusion that, for some reason or other, the male, in virtue of his maleness, is less viable than the female. Under certain circumstances the male, because of a greater inherent fragility, succumbs more easily to the force of death. This phenomenon has been reviewed by Crew (1937), not only for the human species but also for other mammals, birds and insects, both in the open and under controlled conditions of experimentation.

The most obvious explanation of excess male mortality in the medium age groups of the human species is an increased risk for males in the environmental conditions and an increased occupational risk (Martin, 1951). For example, excess mortality in males due to violence is, no doubt, very often due to the fact that males are exposed, or do expose themselves, to a greater extent, to risks of that description. Since, however, during the present century conditions of work have improved considerably as regards safety measures and hygiene, and hours of work have been reduced, one would expect a decrease of excess male mortality on that account, whereas the opposite is the case : excess male mortality is on the increase in our time. Another fact which may be adduced against this type of explanation is that industrial occupation of females is on the increase, and that therefore they are more exposed to occupational risk than before.

Finally there is a pronounced male mortality in the youngest age groups and also in the highest age groups for which the explanation of occupation or environmental risk is obviously not suitable.

The hypothesis of the increased occupational and environmental risk run by males as an explanation for excess male mortality can have only a limited validity. It may explain certain special cases of the phenomenon and it may have application to those ages which expose themselves to risks of dying by acts of violence, and to occupations where predominantly male workers are exposed to the pathogenic influence of mineral or metal dust, gas, fumes etc., but it cannot be regarded as a sufficient explanation of the phenomenon in general (Herdan, 1952).

The phenomenon in question attracted first attention in the form of excess male infant mortality. It was investigated by Lenz (1923) for Germany as a whole, Bavaria, France, Spain, Italy, Austria, Hungary, England, Sweden and Norway. The hypothesis put forward by him was that certain genetic differences between the sexes may be regarded as responsible for excess male mortality.

This hypothesis is based upon the fact that the genetic structure of males, in the human species, is principally different from that of females.

In man, the male is the heterogametic sex, and as such possesses one x-chromosome, whereas the female, as the homogametic sex, possesses two. Thus, the female has two parallel sets of genes whereas the male has, strictly speaking, no such parallel set, because the differential segments of his y-chromosome do not exactly correspond to those of his x-chromosome.

It follows that if a recessive gene for a certain disease or condition is carried in a differential segment of the x-chromosome, it is at once uncovered in the case of the heterogametic individual, and if in its action such a gene is disadvantageous, deleterious or lethal, it will find expression in the phenotype of the individual. If, on the other hand, the individual is homogametic, there is always a chance that the same differential segment which carries the recessive gene in one chromosome may carry in the other chromosome a compensating gene and the expression of the recessive gene in the phenotype would be prevented.

This hypothesis is confirmed by the fact that increase of infant excess male mortality with time, is, in general, accompanied by a decrease of the death rate of infants, males and females together. The argument is as follows: if the lack of resistance to certain diseases were due to certain recessive genes, and if these recessive characteristics were sex-linked, or at least sex-limited, then these diseases would find expression more often as general health conditions improved. As a consequence, the downward trend in the general mortality of infants should be accompanied by an upward trend of excess male mortality. This we find confirmed in all the countries for which we have data. The method is to compare the ratio of male over female infant death rate with the general infant death rate and calculate the correlation coefficient for the two series. One obtains invariably a significant negative correlation, which may be regarded as support for the hypothesis that excess male mortality was due to innate differences between the sexes.

The certainly remarkable phenomenon of the negative correlation between the two series, is not confined to infant mortality but can be extended to mortality for all ages taken together, and thus to the general death rate (Herdan, 1952).

Application to excess male mortality of C.L.

As explained above, a negative correlation between excess male mortality and general health conditions was one of the reasons which led to the genetical hypothesis to account for excess male mortality. For C.L. such a negative corre-

lation can be established between the excess male mortality due to the malignant disease and the mortality for males and females combined due to inflammatory and infectious lung diseases, separately or combined. (See p. 503). This is in full agreement with the curious relation found by Paxon and others between tuberculosis and lung cancer mortality rates (Paxon, 1956 ; Cruickshank 1939 ; Cherry 1924, 1925, 1933). Paxon showed that if the sum of the mortality rates for tuberculosis and cancer of the lung be plotted as a combined mortality rate, then for the past 40 years at least these two diseases have taken together an unchanging toll of lives—2500 in 1910, 2300 in 1920, 2500 in 1930, 2500 in 1950 and 2300 in 1953. This means that for these periods, at least, the ratio :

$$\frac{\text{deaths from phthisis and lung cancer}}{\text{deaths from all causes}}$$

has remained practically constant, which implies that over the period covered, the decrease in the death rate for phthisis has been exactly compensated by an increase in the death rate for lung cancer.

It is now very significant that the emergence of a social class gradient for C.L., and the detailed examination of the Standardized Mortality Ratio for tuberculosis in 1930 and that for C.L. in 1950 should lead to similar conclusions. This, and the fact of C.L. belonging to the class of diseases with pronounced excess male mortality would seem to provide the explanation for the relation observed by Paxon and Cruickshank. In detail, the argument is as follows.

This time it is not the social class gradient in general which affords the explanation, but the detailed pattern of the distribution of the diseases among the occupations.

We compare the 1950 distribution of C.L. among the occupations with the 1930 distribution of respiratory tuberculosis, and find quite a striking similarity in pattern.

The following occupations showed a significantly high Standardized Mortality Ratio for respiratory tuberculosis in 1930 and for C.L. in 1950 : publicans (inn-, hotel-keepers), boot and shoe workers, paper hangers, painters, road transport, iron and steel foundry furnacemen, metal glaziers, polishers, general labourers, boiler makers, platers, messengers and porters, general labourers and other unskilled workers. On the other hand, occupations with a significantly low Standardized Mortality Ratio for respiratory tuberculosis in 1930 and with a significantly low Standardized Mortality Ratio for C.L. in 1950 are : bank and insurance officials, judges, barristers, solicitors, physicians, professional engineers, farmers and their relatives, employers and managers, coal miners conveying material to shaft, coal miners making and repairing roads, other workers below ground in coal mines.

All this may be verified from Tables IV and V and the corresponding graphs of the Standardized Mortality Ratio for cancer of the lung in 1950 and respiratory tuberculosis in 1930, whose construction is explained in the next paragraphs.

Apart from systematic and accidental errors of diagnosis, there is a general type of error to be taken into account in comparing S.M.R's. This is the error due to the fact that the people following a certain occupation form only a comparatively small sample of the total population. Such a sample, if taken at random, may include a greater or smaller number of persons sensitive to the disease in question, or to disease in general. Any increase in the S.M.R. due only to the inclu-

sion of a greater number of sensitive persons must not be put down as due to the occupation or social status. We must, therefore, allow for chance fluctuations in the S.M.R. of a population group before judging its S.M.R. for significance.

There will be a certain probability for a deviation of a specified magnitude to occur due to chance only. The deviation of the S.M.R. from 100 per cent must be considered in the light of these chance deviations. It is with this in mind that the charts (Fig. 1 and 2) have been constructed. The quality control chart method

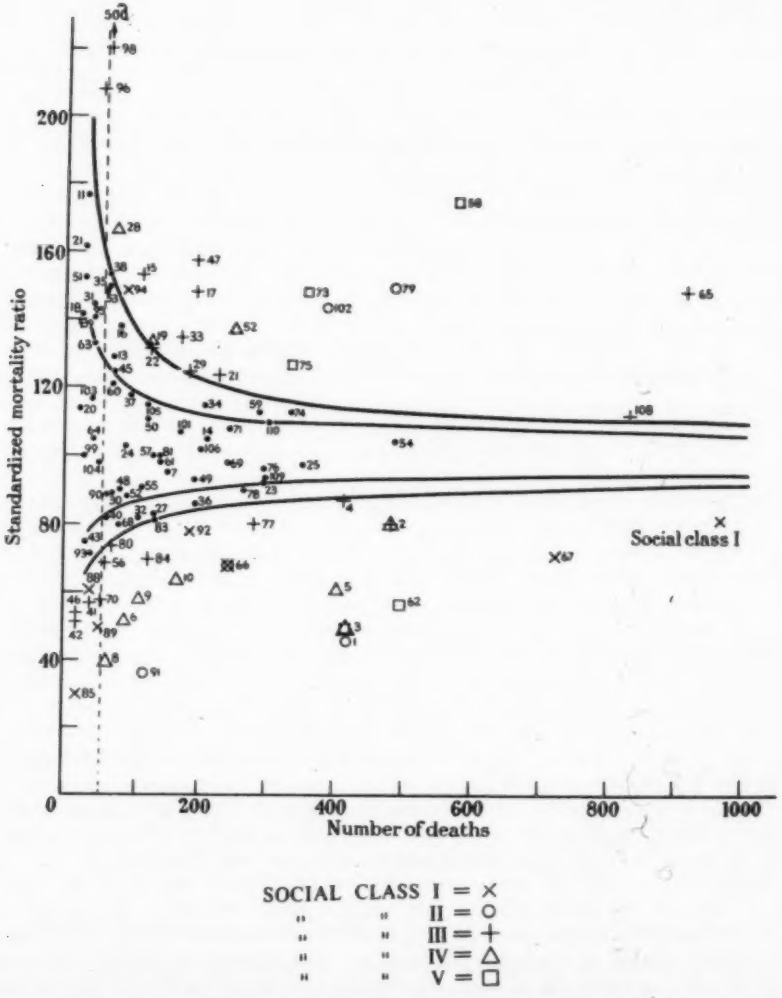

FIG. 1*a*.—Occupational mortality due to cancer of the lung (1949–53).

is here introduced as a device for facilitating the use of the standard error in judging the significance of S.M.Rs.

The general structure of these charts is as follows. A straight line at S.M.R. = 100 represents the S.M.R. for the total population (all males, all married women, all single women, as the case may be) for all causes or for specified diseases. The curved lines are drawn at distances representing 1·5 and 2·5 of the standard error of the S.M.R. (adhering to the practice of the Registrar-General)* and thus including its chance variation in about 14 out of 15 and 160 out of 161 cases respectively on the basis of an assumed normal distribution of the chance variations. According to the size of the random sample (more precisely, according to the number of deaths in the population group) these lines are at different distances from the mean line. Again adhering to the practice of the Registrar-General, they represent the "probable" and "highly probable" borderlines between chance variation and true or real differences in the S.M.R., according to sample size. They can also be regarded as a sort of mathematical gauge by which the significance in S.M.Rs is quickly ascertained. All we have to do is to plot the S.M.R. for the occupation group in question as the vertical ordinate against the total number of deaths in that group during the time under consideration as the horizontal ordinate or abscissa for the cause or causes in question. If the point falls beyond the outer control line (2·5 times the standard error) we can be satis-

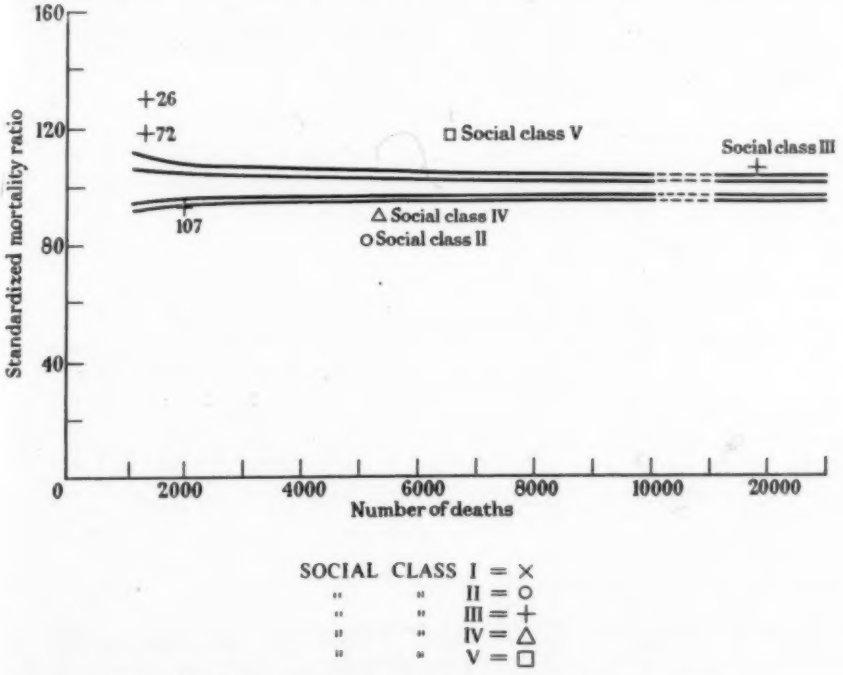

FIG. 1b.—Occupational mortality due to cancer of the lung (1949–53).

* In his publication of 1938.

fied of a real difference and may use it as a reliable pointer to further investigation. If the point falls beyond the inner control limit (1·5 times the standard error) but not beyond the outer control limit, the difference is probably significant and will be worth investigating further.

If the remarkable fact of the similarity in occupational pattern of the S.M.R. for 1930 and C.L. for 1950 is not due to a change in diagnosis, through which the same disease which was characterized by a certain social gradient in 1930 is now diagnosed under another name, that is, if we take the rise in mortality due to C.L. as real, the curious fact of similarity in pattern can be explained through C.L. being one of those diseases which are characterized by excess male mortality. As we have shown, its incidence certainly has all the characteristics of such a disease.

The explanation of the similarity in pattern is then as follows. Occupational groups such as publicans, boot and shoe workers, paper hangers, iron and steel

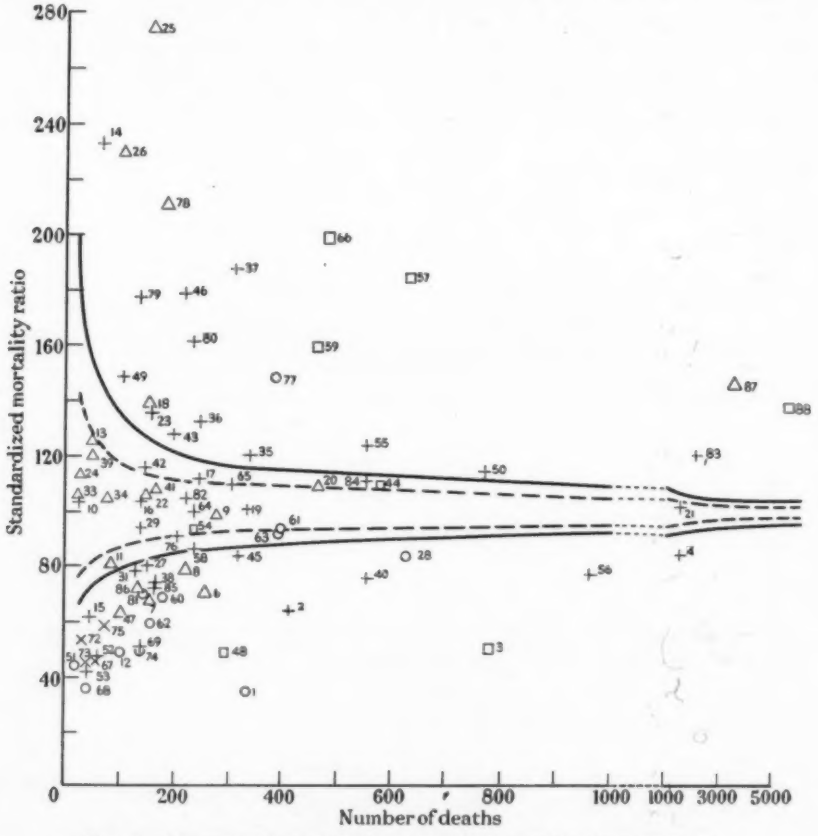

FIG. 2.—Occupational mortality due to respiratory tuberculosis (1930–32). (Symbols as in Fig. 1.)

workers, metal glaziers, boiler makers, platers, messengers, general labourers, who were shown in 1930 to be significantly worse off as regards the incidence of respiratory tuberculosis than the general population must be regarded as specially liable to contracting that disease. Since then the conditions for avoiding and curing infectious and inflammatory lung diseases have greatly improved thanks to improved hygiene, on the one hand, and the sulphonamides, penicillin, streptomycin, etc., on the other, with the result that the mortality due to such diseases

TABLE IV.—*Key to Fig.* 1 *Giving List of Occupations for which S.M.R. is Significantly High and Low* (*Cancer of the Lung* 1949–53)

High			Low		
Social class	No.	Occupation	Social class	No.	Occupation
I	94	Commissioned officers, retired.	I	66	Civil Service executive officers (see also class II).
				67	Secretaries of companies.
				85	Clergymen.
				88	Judges.
				89	Registered medical practitioners.
				92	Professional engineers.
II	79	Retail business proprietors.	II	1	Farmers, farm managers.
	102	Publicans.		66	Civil Service executive officers (see also class I).
				91	Teachers (not music).
III	15	Furnacemen.	III	2	Gardeners (see also class IV).
	17	Moulders.		4	Coalface workers.
	21	Smiths.		41	Spinners, cotton.
	22	Platers.		42	Spinners, wool.
	26	Machine erectors.		46	Grinders.
	27	Drivers of passenger and goods vehicles.		56	Compositors.
				70	Signalmen.
	29	Plumbers.		77	Commercial travellers.
	33	Electrical fitters.		80	Salesmen, shop assistants.
	47	Boot and shoe repairers.		84	Insurance brokers.
	65	Painters and decorators.		107	Clerks, typists (see also class II).
	96	Army — other ranks — retired.			
	98	Royal Navy—other ranks—retired.			
	108	Warehousemen.			
IV	19	Iron foundry labourers.	IV	2	Gardeners (see also class III).
	28	Glaziers, polishers.		3	Other workers in agriculture (see also under class V).
	52	Bakers.		5	Coal miners, undergound (not face workers).
				6	Coal miners (others below ground).
				8	Coal miners (underground road repairers).
				9	Coal miners (others below ground).
				10	Coal miners, surface workers.
V	58	Builders' labourers.	V	62	Other building workers.
	73	Dock labourers.		3	Other workers in agriculture (see also class IV).
	75	Messengers.			

TABLE V.—*Key to Fig. 2 Giving List of Occupations for which S.M.R. is Significantly High and Low (Respiratory Tuberculosis 1930–32)*

High			Low		
Social class	No.	Occupation	Social class	No.	occupation
I	—	—	I	67	Bank and insurance officials.
				72	Judges, barristers, solicitors.
				73	Physicians, surgeons.
				75	Professional engineers.
II	77	Inn-, hotel-keepers.	II	1	Farmers and their relatives.
				12	Employers and managers: gas, bricks, chemicals.
				28	Employers and managers in Occupation Orders VIII-XXI, XXXI.
				51	Railway officials.
				60	Retail proprietors, etc., e.g. grocery.
				62	Wholesale proprietors, etc.
				68	Civil Service and Local Authority: administrative and executive.
				74	Teachers (not music).
				81	Draughtsmen, costing clerks, etc.
III	14	Potters, ware makers, etc.	III	2	Gardeners, nurserymen, florists.
	35	Makers of textile goods.		4	Coal hewers and getters.
	36	Boot and shoe makers, repairers.		15	Workers in chemical processes.
	37	Boot and shoe workers and factory operatives.		27	Plumbers (not chemical plumbers).
	43	Printing machine minders, printers, etc.		31	Textile weavers (cotton).
	46	Masons.		38	Bakers and pastrycooks.
	49	French polishers.		40	Carpenters.
	50	Paper hangers, painters.		45	Bricklayers.
	55	Road transport: horse drivers.		52	Railways: engine drivers.
				53	Railways: signalmen.
	79	Waiters.		56	Road transport: motor drivers.
	80	Hairdressers.		69	Police.
	83	Typists and other clerks (other than Civil Service).		85	Stationary engine drivers not underground in mines.
IV	18	Iron and steel foundry furnacemen.	IV	6	Coal miners: conveying material to shaft.
	25	Metal grinders.		7	Coal miners: making and repairing roads.
	26	Metal glaziers, polishers, etc.		8	Coal miners: other workers below ground.
	78	Barmen.		47	Platelayers.
	87	General labourers.		86	Boiler firemen and stokers.
V	23	Boiler-makers, platers and iron shipwrights.	V	3	Agricultural and gardeners labourers, etc.
	57	Water transport: dock labourers.		48	Navvies in building trade, etc.
	59	Messengers and porters.			
	66	Costermongers, newspaper sellers.			
	88	General labourers and other unskilled workers.			

has dwindled to negligible proportions compared with what it was. But this had as a consequence that recessive genes conducive to contracting lung disease were, so to speak, coming into their own, especially in the male where the chance of a compensating gene, that is one with a counteracting tendency, is missing.

It is true that among people who have never smoked, nor lived in large towns, there appears to be no excess male mortality from C.L. (Stocks, 1958). This, however, is not at variance with the genetical hypothesis put forward. The non-smokers who have never lived in large towns are a selected part of the population, not only as regards residence, but probably also as regards genetical constitution. Their genetical make-up may be predominantly free from recessive genes with lethal action finding expression more readily in the male, and in this case the phenomenon of excess male mortality would not materialize.

If our conclusion is correct, there should be a pronounced negative correlation between the mortality due to inflammatory and infectious lung diseases on the one hand, and excess male mortality ratio due to the malignant disease on the other. As Table VI shows, this is definitely the case. The correlation is negative and highly significant.

TABLE VI.—*Correlation Between Mortality Due to Inflammatory Lung Disease and T.B. and Excess Male Mortality Ratio Due to Malignant Disease*

	Comparative Mortality Index for respiratory tuberculosis and pneumonia, males and females combined	Excess Male Mortality Ratio for C.L.
1937	1·13	1·02
1938	1·00	1·00
1939	0·92	0·96
1940	1·10	1·02
1941	1·08	1·08
1942	0·92	1·07
1943	1·00	1·16
1944	0·88	1·19
1945	0·86	1·18
1946	0·77	1·25
1947	0·80	1·26
1948	0·67	1·29
1949	0·69	1·34
1950	0·57	1·39
1951	0·61	1·48
1952	0·49	1·53
1953	0·49	1·52
1954	0·43	1·65
1955	0·45	1·67
1956	0·44	1·71

$r = -0·885$

The particular occupations which were significantly worse as regards the inflammatory and infectious diseases are so widely different that this tendency cannot be strictly called an occupational risk : it must lie more with the genetical make-

up of these groups, and their being genetically susceptible to lung disease makes it appear natural that they should contract C.L. as an alternative disease as soon as the older ones such as tuberculosis and pneumonia had given way to successful treatment.

The somewhat lengthy exposition of the argument in Section II makes it advisable to state it now once more briefly. The mortality due to C.L. has been rising steadily in the last decades, and so has the excess male mortality due to that disease, the death rate of males being always in every year in excess of that for females. If the disease was due to a recessive gene which is uncovered in the case of the heterogametic individual, it will find more often expression in the phenotype of the male individual. In other words, if C.L. has a genetic foundation, then due to males being the heterogametic sex it will have a greater chance of appearing in the phenotype of the male, and the more so the greater the improvement in general conditions for diseases of the lung. As a consequence, the upward trend of excess male mortality due to C.L. should be accompanied by an equally pronounced downward trend in the general mortality, males and females combined, due to other lung diseases. This is actually the case. Thanks to the action of the sulphonamides, penicillin, streptomycin, etc., general mortality for males and females combined due to pneumonia and tuberculosis of the lung has decreased not less spectacularly than that due to C.L. has increased in the last 30 years or so. The correlation between the Comparative Mortality Index due to pneumonia or tuberculosis of the lung combined for males and females on the one hand, and the ratio of male to female mortality due to C.L. on the other, is highly significantly negative, in full agreement with the genetical theory of excess male mortality.

To the general disadvantage at which the male part of the population is subject, through recessive genes with lethal action finding expression more easily in the male phenotype, there must be added the selective action of the Great War, and also of the last war to some extent, upon the male population of this country. Since it is mostly the healthy male population which is under arms in war-time, the number of killed and wounded in war-time amounts to a reduction, to that extent, of the healthy male population of the country. The excess male mortality, for certain age groups at least, may be the consequence of the elimination of that part of the healthy male population, the remaining part not being so resistant to disease as those who were eliminated.

To account for the spectacular excess male mortality of a disease which, like C.L., is not obviously sex-linked, we have thus two facts : an equally spectacular improvement in mortality due to epidemic and inflammatory lung diseases, males and females combined, which in itself is conducive to bringing to the fore the action of recessive genes with lethal effect in the males as heterogametic individuals, and the selective action of war tending to reduce in numbers that part of the male population whose genetical make-up renders it more resistant to disease.

Both the facts and the hypothesis discussed in this paper suggest new lines of research with a view to settling the controversial problem of the association between smoking and C.L. It would seem now advisable to compare the smoking habits in samples from the occupations above the outer control line of Fig. 1 with the smoking habits of the occupations below the outer control line, that is of the occupations which are significantly worse off as regards the disease than the general population, and the occupations which are significantly better off.

Another line of research suggested by our results is that into lung disease of all kinds among the relatives of C.L. sufferers.

SUMMARY

The emergence of a definite social class gradient for C.L. in the latest publication of the Registrar-General (1958) appears to throw light upon the nominal increase in mortality due to C.L. The hypothesis of a change in diagnosis as accounting for the emergence of a social class gradient for C.L. between 1930 and 1950 is discussed in Section I. In Section II the rise in mortality due to C.L. is taken to be real, but again the appearance of a social class gradient points to an interpretation different from the current hypothesis about smoking and cancer. The steady rise in excess male mortality for that disease is accompanied by an equally spectacular fall of mortality of both sexes for inflammatory and infectious lung diseases, especially respiratory tuberculosis.

It is, moreover, shown in detail that the relationship of the two diseases, C.L. and respiratory tuberculosis, is such that certain occupations which were characterized by an excessively high or excessively low Standardized Mortality Ratio for tuberculosis of the lung in 1930 have now an excessively high or low Standardized Mortality Ratio for cancer of the lung, whereas their tuberculosis mortality is greatly reduced. A similar compensatory relation between the two diseases was established by Paxon (1956) who adds, however, that his views are based on correlations which may be entirely fortuitous. Such a view appears too modest in the face of all the facts. The relationship between two diseases as shown here to exist between respiratory tuberculosis and C.L., according to which their combined mortality rate should remain sensibly constant, is quite unique and there is no other pair of important diseases in which a similar movement can be detected in the rates. Considering that the combined mortality rate may be written as the sum of the two single mortality rates, we can formulate the result by saying that the alternative probability that a person should die of either respiratory tuberculosis or C.L. remains sensibly constant in the population.

In general, if we were to find in science a relation of this sort between two variables, we would not be content with regarding it as merely fortuitous, but would look for an explanation. The explanation put forward here is that it was the action of the sulphonamides, penicillin, streptomycin, etc. resulting in the reduction of the combined mortality of males and females due to pneumonia and tuberculosis of the lung, which is responsible for bringing to the fore a disease which has its roots, partly at least, in the genetical make-up of man. Since, according to the genetical theory of excess male mortality the human male is at a disadvantage in this respect, this would account for both the fact of excess male mortality due to C.L. and the negative correlation between excess male mortality and mortality due to respiratory tuberculosis and pneumonia, males and females combined. To the genetical disadvantage at which the male part of the population is subject through recessive genes with lethal action finding expression more easily in the male phenotype, there must be added the selective action of the Great War, and also of the last war to some extent, upon the male population of this country. The excess male mortality for certain age groups, at least, may be the consequence of the elimination of a considerable part of the healthy male population, the remaining part not being so resistant to disease.

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THE SMALL TUMOUR-LIKE LESIONS OF THE KIDNEY

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EVERYONE performing routine post-mortem examinations finds from time to time a small nodule in either the cortex or the medulla of the kidney. Microscopy of a cortical lesion usually reveals a minute papillary adenoma, of a medullary one an area of fine fibrosis. Other types of nodule are less common. Newcomb (1936) and Apitz (1943) have made careful studies of small nodules found beneath the capsule in the hope of throwing light on the pathogenesis of malignant renal neoplasms. Zangemeister (1936) has studied the medullary lesions and discussed their possible nature. We are presenting the results obtained by examining the nodules found in a number of kidneys removed at routine necropsy.

MATERIALS AND METHODS

A single kidney was taken from each of 212 bodies. The side from which it came was not recorded. Most of the bodies had been dissected at the request of the Coroner, a few came from hospitals. Seventy-nine were females and 133 were males. The age and sex distribution is shown in Table I. Apart from discarding decomposed organs, there was no selection.

TABLE I.—*The Age and Sex Distribution of the Cases*

	0–10	11–20	21–30	31–40	41–50	51–60	61–70	71–80	81–90	91–100
Male cases . .	0	9	9	7	12	22	36	29	9	0
Female cases . .	1	1	1	1	3	9	16	32	14	1
Total cases .	1	10	10	8	15	31	52	61	23	1

The whole kidney with its capsule intact was fixed for at least two weeks in 10 per cent formol saline. It was then bisected in the transverse plane. Each half kidney, cut surface downwards, was placed on a Berkel's electric ham slicer and successive transverse slices were cut, the kidney being pressed against the carrier of the machine by a block of soft wood. Slices were kept in their proper order and examined serially in a good light.

Blocks for histology were taken from all slices showing solid nodules. Cysts, scars and prominent vessels were discarded after examination with a hand lens. The optimum thickness of the slices was found to be 0·12 cm., which was the thinnest block which could be processed without curling. It was recognised that lesions of less than 0·1 cm. in diameter might be lost but as the great majority of nodules was between 0·1 and 0·2 cm., this was not considered a serious objection to the method. In any case the search for really minute lesions was unrewarding as

they were often lost in the processing. The slices varied in thickness from 0·10 to 0·15 cm., with an average of 0·123 cm., and in 180 of the cases they were 0·13 cm. or thinner. In order to make sure that the side of the block of tissue bearing the lesion was embedded face downwards for sectioning, a method had to be adopted to render the block asymmetrical in such a way that it could be recognised by the technician processing it. The block was placed with the lesion uppermost and notches were cut in two adjacent edges at right angles. Two notches were made in one edge, and a single notch in the other to its right. Finally the blocks were embedded in paraffin and 5 μ sections were cut and stained with haemalum and eosin.

FINDINGS

The 212 kidneys examined yielded a total of 265 lesions after discarding a small number of cysts or thickened vessels which had been sectioned by mistake. These lesions were contained in only 115 of the kidneys; no nodules were discovered in the other 97. Only those lesions which could be examined histologically have been discussed, some 35 lesions were lost but many of these were minute streaks in the cortex or vague areas of fibrosis in the medulla.

Table II sets out the histological types of lesion found. Further description and discussion will be limited to the first four categories. Of the remainder, nothing further need be said.

TABLE II.—*Histological Types of Lesion Found in 212 Kidneys*

Lesions	Total Number	Number of kidneys	Maximum number per kidney
Medullary fibrous nodules	159	78	8
Cortical adenomas	49	31	6
Lipomas, myomas or mixed lipo-myomas	31	25	3
Adrenal rests	10	10	1
Neurofibroma of pelvis	1	1	1
Cancer metastases	2	2	1
Tubercles	1	1	1
Calcified nodules	1	1	1
Pyelonephritic abscesses	11	5	5

Cortical Adenomas

A total of 49 small adenomas of the cortex were found in 31 kidneys. In one kidney there were 6 such lesions, in another 5, in a third 4, and in a fourth 3. Four kidneys bore 2 adenomas each, the remaining 23 a single one. The age and sex distribution is shown in Table III. A woman of 50 was the youngest to have a tumour, a man of 57 had the greatest number.

TABLE III.—*The Age and Sex Distribution of Cortical Adenomas*

	0–10	11–20	21–30	31–40	41–50	51–60	61–70	71–80	81–90	91–100
Male cases	0	0	0	0	0	10	10	5	1	0
Female cases	0	0	0	0	1	1	0	3	0	0
Total cases	0	0	0	0	1	11	10	8	1	0
Total lesions	0	0	0	0	1	21	11	14	2	0

All the adenomas in this series were small, the largest measured 0·3 × 0·2 cm. Ten tumours had a greatest diameter of 0·2 cm. or more, 37 were over and 12 under 0·1 cm. diameter. There were probably many smaller ones which were lost or overlooked. None of these tumours was encapsulated. Most of them had very irregular outlines and the tumour tissue insinuated itself freely between the tubules and glomeruli of neighbouring nephrons. Following Newcomb (1936) we distinguished three histological types. In our series of 49 adenomas, 23 were small-celled, 20 were foam-celled and 6 were eosinophilic. The nuclei of all the adenomas were of similar appearance. Although darker staining than the nuclei of the convoluted tubules, they were the same shape and size and were completely regular. No mitoses were seen and the nuclear regularity made it easy to pick out a small metastasis of a well differentiated adenocarcinoma of the uterus from a series of adenomas. The small-celled variety of adenoma had scanty cytoplasm and the cells were nearly always arranged in tubules with occasional papillary ingrowths (Fig. 1). The foam-celled type had more abundant cytoplasm, which was foamy and closely resembled the cytoplasm of hypernephroma cells. These tumours were usually papillary (Fig. 2) and very frequently showed foci of calcification. The nodules of calcium were laid down in concentric rings (Fig. 3). Rarely this type of adenoma was solid (Fig. 4). The eosinophilic adenoma had even more cytoplasm than the foamy type and this cytoplasm stained a uniform bright pink with eosin. These tumours were all papillary (Fig. 5).

Discussion

Most pathologists who have studied these tumours agree that they are found more frequently in the elderly. Weichselbaum and Greenisch, cited by Ewing (1928), found adenomas limited to subjects over 30 steadily increasing in number with age, and present in 10 per cent of all those over 80. Trinkle (1936) pointed out a close parallel between the age incidence of adenomas and hypernephromas. In his series of adenomas, the average age was 62, and the average age of 108 hypernephromas collected by him from the necropsy records of the University of Minnesota was 61 years. Apitz (1943) found in his series that almost 20 per cent of men over 44 had one or more adenoma, but only half that number of women. The women who developed them, however, tended to do so at a younger age. In Table II it will be seen that all our cases occurred in the middle-aged and elderly and that they were more common in men than women. The increase in the number of these tumours as age advances is evidence that they are neoplasms rather than malformations.

The relationship of adenomas to scars of the renal cortex is worth examining as it may shed light on the pathogenesis of these tumours. All the kidneys we examined which bore adenomas contained some scars, but the same could be said of all the other kidneys from subjects over 50. A few of our adenomas appeared in obvious scars but the majority did not. However, in both our cases of 5 or more tumours, the kidneys were severely granular with cysts. Apitz (1943), who cut serial sections of all his 725 adenomas, was able to prove statistically that scarring played a part in their pathogenesis. He found that kidneys with more than 4 adenomas usually had severe scarring. Of interest was one of his cases where a contracted kidney contained 26 adenomas. The kidney on the other side was healthy and bore only one tumour. Nevertheless, adenomas were often indepen-

dent of scars and some scarred kidneys were without adenomas. Trinkle (1936) concluded that adenomas occur most often in kidneys with vascular disease and in the advanced years of life, and suggested that they resulted from the proliferation of tubules whose blood supply was cut off. In the majority of cases, the tubule atrophied but occasionally it underwent hyperplasia. The hypothesis of adenoma formation as the result of attempted regeneration of a damaged nephron is a very interesting one and although some evidence favours it, we do not regard it as proved. Adenomas have been produced experimentally in animals. Zollinger (1953) gave rats weekly injections of 20 mg. of lead phosphate. The earliest change in the kidneys was cyst formation accompanied by an irregularity and hyperplasia of the tubular epithelium. In 19 of the 29 rats which survived this treatment for 10 months or more, adenomas, papillomas and cystadenomas of the renal cortex developed, which in 3 cases became malignant. Histologically these tumours looked like the various types of adenoma of the human renal cortex. Sempronj and Morelli (1939) also produced " cystic nephritis with multiple adenomas and hypernephromas " by injecting β-anthroquinoline.

It would be inappropriate here to review in detail the controversy of the origin of the clear-celled carcinoma of the kidney which is dealt with in a masterly way by Nicholson (1950). However, few critical pathologists since Sudeck (1893), Stoerk (1908) and Glyn (1912) believe with Grawitz (1883) that the " hypernephroma " is a carcinoma of an adrenal rest in the kidney. It is remarkable how hard this belief has died and Apitz in 1943 after examining his 725 adenomas seemed to favour it. In our opinion the hypernephroma resembles the foamy-celled adenoma much more closely than it resembles the adrenal rest. Although the hypernephroma is usually solid rather than papillary, search of such a tumour will often reveal villous areas. Nicholson (1909) observed that a solid hypernephroma after removal recurred with a villous structure. He pointed out that a solid organ like the suprarenal could not produce villi. The best summary of the relation between adenoma and hypernephroma is given by Willis (1953). " Are adenomas related to carcinomas ? Anyone who has examined and compared the structure of these tumours will have no hestitation in joining Newcomb and Trinkle in a strongly affirmative answer. Indeed a sharp separation of adenomas and carcinomas is not possible. Some adenomas show a structure indistinguishable from that of carcinomas and it is purely a matter of opinion whether we regard such tumours as typical adenomas or as young carcinomas which we happen to have discovered before they have metastasised. Many an adenoma found incidentally at necropsy differs not one whit from some of those small symptomless carcinomas which have produced precocious metastases. Renal tumours, like other tumours, differ in their individual rates of growth, invasiveness and metastasising proclivities. It is proper that those tumours which for long periods grow slowly, attain a highly differentiated structure and fail to spread should be called adenomas to distinguish them from their more active fellows."

Franks (1954), making a careful study of prostate glands from elderly subjects, found a high proportion of them contained nodules which histologically closely resembled carcinoma, though there were no metastases. Similar lesions have been found in the lungs, often in relation to scars, by Raeburn and Spencer (1953), in the liver, thyroid, and gastrointestinal tract. These tumours have been called " latent carcinoma " and it seems likely that adenomas of the renal cortex belong to this category. The rate of growth of hypernephromas is very variable. Botsztejn

and Zollinger (1948) report a case of one which produced early metastases but remained symptomless for 8½ years, with a very slow rate of growth. Perhaps the cortical adenoma is such a tumour which has grown to the size of 0·2 cm. and then remained latent.

Adrenal Rests

A total of 10 adrenal rests were found in our series of 212 kidneys. The age and sex distribution is shown in Table IV.

TABLE IV.—*The Age and Sex Distribution of Adrenal Rests*

	0–10	11–20	21–30	31–40	41–50	51–60	61–70	71–80	81–90	91–100
Male cases . .	0	1	0	0	1	1	3	1	0	0
Female cases . .	0	0	1	0	0	1	1	0	0	0
Total cases .	0	1	1	0	1	2	4	1	0	0
Total lesions .	0	1	1	0	1	2	4	1	0	0

To the naked eye, all the adrenal rests were bright orange-yellow in colour. They were all on the surface of the kidney at the upper pole and external border. There was one large mass of adrenal 3 × 2 cm. in area and 0·5 cm. thick, which was mainly extracapsular and extended beneath the capsule in one place only. All the others were small and were placed immediately beneath the capsule, sometimes extending into the layers of the capsule itself. They were all flattened, with a diameter of up to 0·6 cm. Most of them were not more than 0·1 cm. thick, but one was 0·3 cm. thick and extended right through the capsule. They were not themselves encapsulated and in some cases the adrenal cortical tissue enclosed portions of nephron, or even adipose cells (Fig. 6). The rests consisted of normal-looking adrenal cortical tissue of the zona glomerulosa and zona fasciculata (Fig. 7). No medullary tissue was present in any of the rests examined.

Discussion

There seems to be little doubt that these small masses are ectopic suprarenal cortical tissue and are not neoplastic in nature. Albrecht (1904) gave the name Choristoma to a tumour-like formation which can be regarded with certainty as part of an organ displaced to an abnormal position only to give the impression of being a tumour by virtue of its abnormal position and its contrast against its surroundings. These nodules fit this definition and in view of the popularity of Albrecht's word " Hamartoma " it is interesting to note his other word " Choristoma ". Their histological appearance is completely unmistakable and they do not in the least resemble cortical adenomas. The numbers in our series are small but Table IV shows that they appear at all ages and probably equally in both sexes. The incidence in our series is 4·7 per cent for *one* kidney, which is in conformity with the findings of Apitz (1943), who carefully examined the surfaces of both kidneys in a series of 4309 necropsies and had an incidence of 7·33 per cent of adrenal rests. Bilateral adrenalectomy and oophorectomy is nowadays commonly performed on patients with advanced breast cancer. It is only successful in a proportion of cases and when regression of metastases does occur, it is rarely for longer than 2 years. If over 7 per cent of the patients have intrarenal ectopic suprarenal

tissue and perhaps an even greater number have adrenal rests elsewhere, this may account for many of the failures of this operation.

Lipomas, Myomas and Mixed Lipomyomas

There was a total of 31 of these nodules, of which 12 were pure leiomyomas, 7 pure lipomas, and 13 mixed. Three were in the medulla, the rest in the cortex. They occurred altogether in 25 kidneys. One kidney bore 3 nodules (one lipoma and 2 myomas), 4 kidneys bore 2 and the remainder one each. The age and sex distribution is shown in Table V. Of the 5 tumours in men, 2 were myomas and 3 were mixed.

TABLE V.—*The Age and Sex Distribution of Lipomas and Myomas*

	0–10	11–20	21–30	31–40	41–50	51–60	61–70	71–80	81–90	91–100
Male cases	0	0	0	0	0	0	3	2	0	0
Female cases	0	0	0	1	0	4	6	8	1	0
Total cases	0	0	0	1	0	4	9	10	1	0
Total lesions	0	0	0	1	0	4	10	14	2	0

The largest nodule measured 0·6 × 0·9 cm. and was of the mixed type containing an arterial malformation, 11 were 0·3 cm. in diameter or more, and the rest were between 0·1 and 0·3 cm. These nodules therefore tended to be a little larger than the others of the series. The outline of the nodules was irregular and in no case was there a capsule. The pure lipomas consisted entirely of adult adipose tissue (Fig. 8). They were confined to the cortex. The pure leiomyomas were made up of interlacing bundles of smooth muscle cells. Four of them were subcapsular and firmly attached to the capsule, but they in no way differed from the others in their architecture (Fig. 9). The mixed tumours consisted of adipose tissue with a varying amount of smooth muscle bundles between the groups of cells (Fig. 10). Examining the series it was impossible to escape the conclusion

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EXPLANATION OF PLATES

FIG. 1.—Small-celled adenoma. H. and E. × 230.

FIG. 2.—Papillary foam-celled adenoma. H. and E. × 230.

FIG. 3.—Foam-celled adenoma with calcified nodule. H. and E. × 230.

FIG. 4.—Solid foam-celled adenoma. H. and E. × 230.

FIG. 5.—Eosinophilic adenoma. H. and E. × 230.

FIG. 6.—Adrenal rest partly outside and partly beneath the renal capsule. Note the included fat cells. H. and E. × 40.

FIG. 7.—Subcapsular adrenal rest. H. and E. × 50.

FIG. 8.—Pure lipoma. H. and E. × 50.

FIG. 9.—Subcapsular leiomyoma. H. and E. × 50.

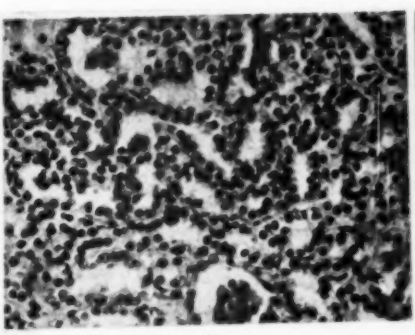
FIG. 10.—Mixed leiomyo-lipoma. H. and E. × 50.

FIG. 11.—Fibrous nodule with many fibrocytes. H. and E. × 50.

FIG. 12.—Fibrous nodule with fine fibres running in all directions. H. and E. × 50.

FIG. 13.—Fibrous nodule with tubules running through. H. and E. × 50.

FIG. 14.—Fibrous nodule with hyaline. Irregular tubes of hyaline surround atrophic collecting tubules. H. and E. × 50.



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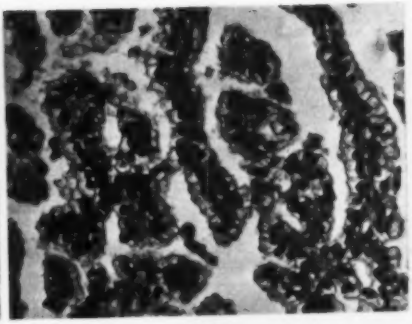

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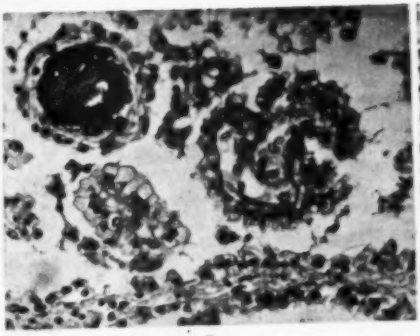

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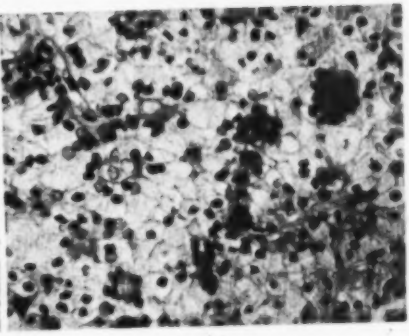

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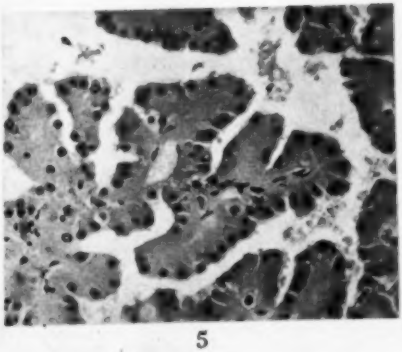

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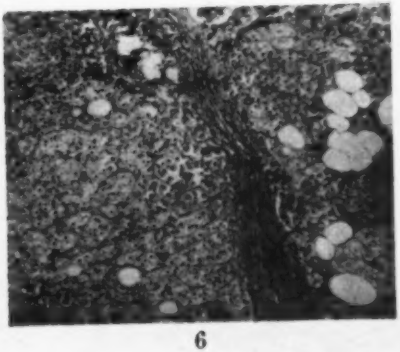

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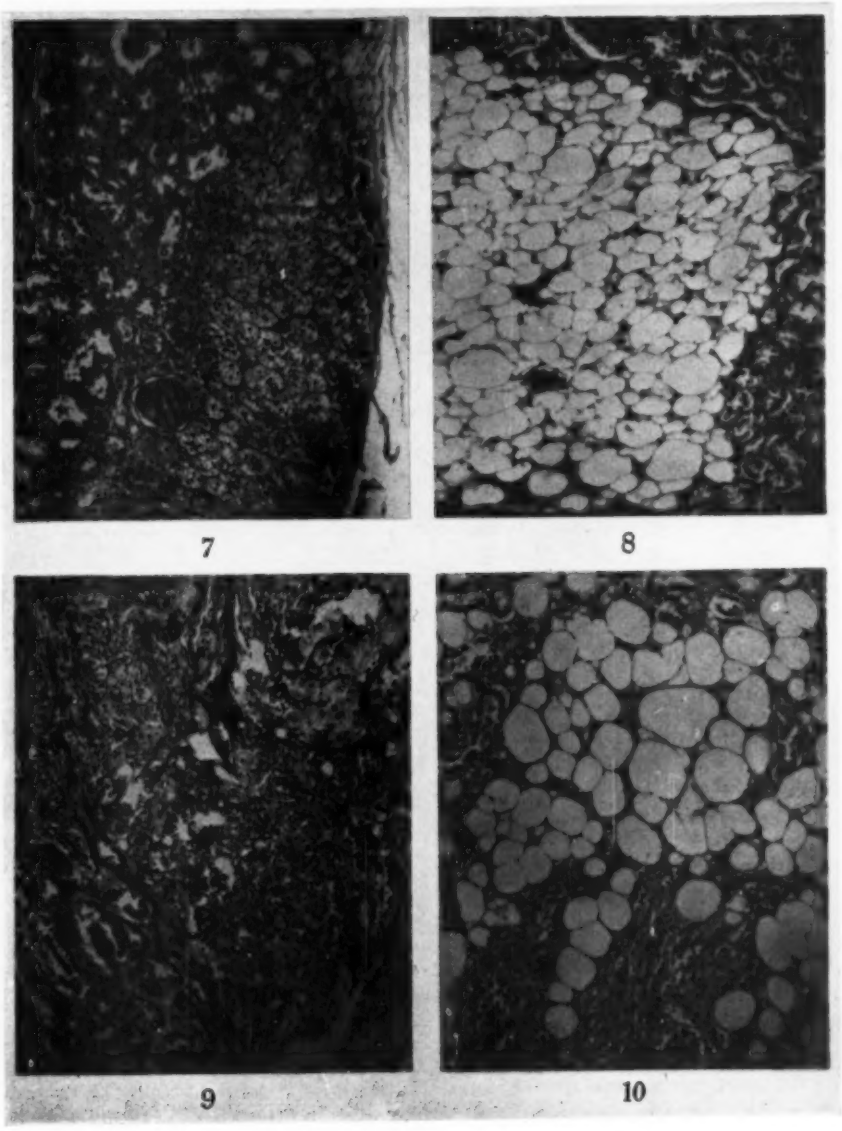

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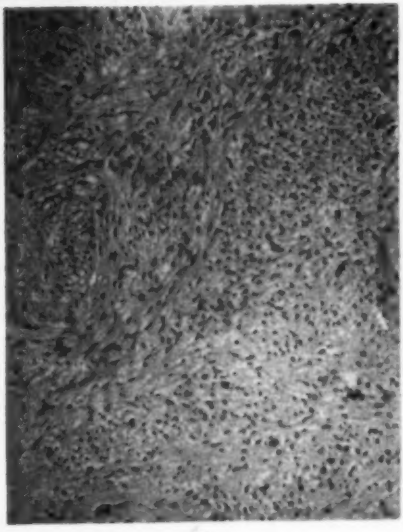

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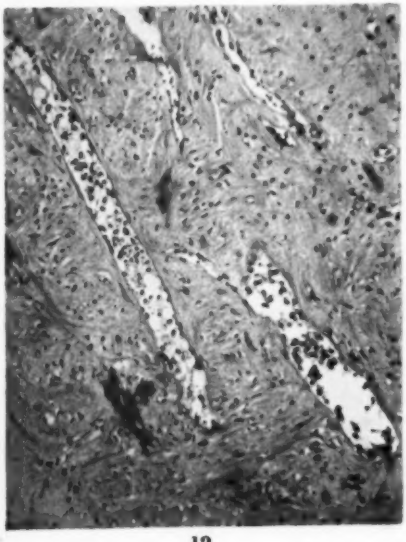

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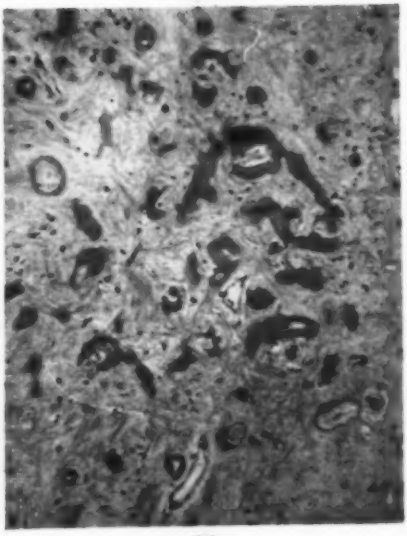

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that these three types were varieties of a single species of lesion with every gradation between them ; where a single kidney bore more than one, they often differed greatly in fat or muscle content. Van Gieson staining of the muscular elements showed a few collagen fibres but the tissue was predominantly muscular.

Discussion

Albrecht (1904) gave the word "Hamartoma" to a tumour-like malformation in which there was nothing more than an abnormal mixture of the normal tissue components of the organ. The abnormality might be in quantity or in dislocation or in respect of the grade of developmental maturity, or in all three of these respects. The small tumour-like masses we have described give no appearances of being independent neoplastic growths and they appear to fit Albrecht's definition of hamartoma. The tissues are a mixture of fat, smooth muscle and occasionally vessels. They appear to have been dislocated from the renal capsule or perhaps the pelvis. Smooth muscle is found in foetal but not in adult renal capsule so that an abnormality of developmental maturity must be the reason for its presence here. Unfortunately we have only one case appearing before middle age but large tumours of fat and muscle have been reported in children. Cottrell and Heckel (1954) described a baby girl of 3 months with such a tumour 5 × 2 cm. in size. A very large leiomyoma in a girl of 15 was described by Gordon, Kimmelstiel and Cabell (1939), who give a very good review of the literature. This incidence of such lesions in the young supports the idea of their being hamartomas, or in the case of the larger tumours, neoplasms arising in a hamartoma. Their presence in tuberose sclerosis (Hulse and Palik, 1951) also supports this view as in this disease such abnormalities are present in other organs. A finding which we are quite at a loss to explain is the much greater frequency in our series of this lesion in women. It is not sufficient to suggest that lipomas are mere adipose infiltrations and that women are obese more frequently than men. Pure myomas and mixed tumours were also much more frequent in women.

Medullary Fibrous Nodules

A total of 159 fibrous nodules of the medulla were found in 78 of the kidneys examined. One kidney had 8 such nodules, 6 kidneys had 5, 7 had 4, 8 and 3, 13 had 2 and in 43 kidneys only a single nodule was found. The age and sex distribution is set out in Table VI.

TABLE VI.—*The Age and Sex Distribution of Medullary Fibrous Nodules*

	0–10	11–20	21–30	31–40	41–50	51–60	61–70	71–80	81–90	91–100
Male cases . .	0	0	1	1	3	9	11	15	5	0
Female cases . .	0	0	0	0	1	3	5	15	9	0
Total cases .	0	0	1	1	4	12	16	30	14	0
Total lesions .	0	0	1	2	8	21	42	55	30	0

The largest lesions, of which there were 17, were up to 0·3 cm. in diameter. There were 36 nodules of 0·2 or 0·25 cm., 91 of 0·1 or 0·15 cm. and 15 which were less than 0·1 cm. diameter. A number of the smaller lesions might have been

lost or overlooked. These nodules were usually spherical in shape, occasionally oval. They consisted of fine fibrous tissue with a varying number of fibrocytes (Fig. 11). The fibres showed a " ball of twine " arrangement unlike the parallel fibres of the normal medullary connective tissue (Fig. 12). Most of the lesions had a few tubules running through them (Fig. 13). Although the majority of the nodules appeared to the naked eye as white, clearly defined tumours, there was no sharp edge to them when they were examined microscopically and they were never encapsulated. They did not contain smooth muscle and the occasional myoma of the medulla was easily distinguished. One-third of the nodules showed areas of a curious hyaline change in the connective tissue. The hyaline material stained a uniform bright pink with eosin and failed to give a red colour with Van Gieson. It was arranged in irregular streaks or in rounded tubes (Fig. 14).but it was obviously changed collagen and not thickened basement membrane because typical collagen fibres could be seen entering or leaving hyaline masses. In non-hyaline areas the collagen fibres formed around the remains of tubules so it was not remarkable that when collagen became hyaline it would take up their shape. In a few of the nodules, the ground substance between the fibres was mucoid and stained bluish with haematoxylin, an appearance often common to several nodules in the same kidney.

Discussion

These nodules were first described by Virchow (1863), who regarded them as inflammatory in origin. He said that there existed a circumscribed *nephritis interstitialis tuberosa* which was focal and gave place to fibrous tumours formed by progressive hyperplasia of the interstitial tissue, at the same time the collecting tubules contained in these areas underwent atrophy little by little. This view was questioned by Albrecht (1904), who said that there was no doubt that they were genuine hamartomas which differed from the normal only in the presence of an abnormal amount of connective tissue having a typical arrangement. The regular disposition of the tubules made it impossible to regard them as tumours in the strict sense, the connective tissue was laid down in a regular relation to the tubules, there was no sharp edge to the fibroma and no evidence of growth after completion of the growth of the kidney. He proposed, therefore, the name *hamartoma fibro-canaliculare renis* for these formations. Genewein (1905), who reviewed the literature on these lesions, followed Albrecht and called them hamartomas. The only recent study is that of Zangemeister (1936), who cut 99 pairs of kidneys on a ham slicer, 11 from the newborn and 9 or 10 from each subsequent decade. He found that 56 per cent of men and 60 per cent of women bore these lesions. The youngest was 18 years old and there was a constant increase with age, with an incidence of 80 per cent in the fifth decade. He distinguished three stages in the development of a nodule. Young lesions were rich in cells with a fine fibrillary skein-like arrangement of the collagen. Nodules in the second stage were moderately rich in cells with coarse fibres and perhaps a little hyaline, while old lesions were poor in cells, the connective tissue was split up by fissures and hyaline was abundant. He was able to show that the younger cases tended to have young lesions and the older cases old lesions. He concluded that the fibroma of the renal medulla was not a hamartoma in Albrecht's sense but was a fibrosis dependent on the generally increased tendency to tumour formation as age advanced. He compared it to verruca senilis or mucous polyp of the intestine.

These masses show little tendency to growth and do not appear to give rise to neoplasms. The large tumour described by Clar (1933) at the upper pole of the kidney in a man of 30 appeared to be a myoma and the hilar tumour in a man of 38 reported by Kretchmer (1932) a neurofibroma. There is no definite relationship between old infarcts or old pyelonephritis and these lesions. We have found no evidence of an inflammatory origin postulated by Virchow and we are inclined to favour the view of Zangmeister, comparing them perhaps to keloids rather than to polyps.

SUMMARY

More than 200 kidneys removed from routine necropsies were cut on a ham slicer. All small nodules found were examined microscopically and with a few exceptions they fell into one of four categories. Suprarenal rests and small adenomas were found in the cortex, small fibrous lesions in the medulla, and in either cortex or medulla there were a number of lesions consisting of adipose tissue, smooth muscle or a mixture of these.

The suprarenal rests were regarded as ectopic suprarenal cortical tissue and were always on the surface of the organ. They were present in 5 per cent of the kidneys examined.

The cortical adenomas became commoner as age advanced. When many were present in a single organ there was usually severe scarring as well, but their relation to scars was inconstant. They were considered as neoplastic in origin though not actively growing and their relationship to hypernephromas was discussed.

Fibrous lesions of the medulla were also more common in the elderly. For this reason they were not considered to be hamartomas but focal overgrowths of fibrous tissue, perhaps like keloids, in response to an unknown stimulus.

Leiomyomas, lipomas and mixtures of these were all regarded as variants of the same lesion, a hamartoma. They were present at all ages and for some reason were commoner in women than in men.

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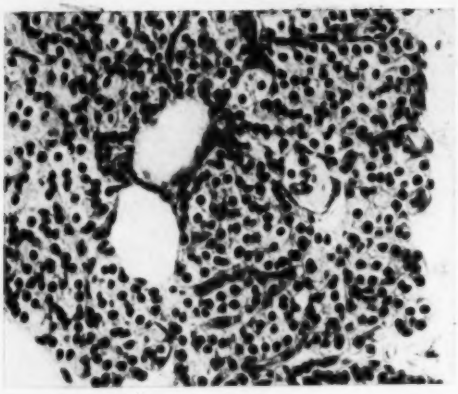

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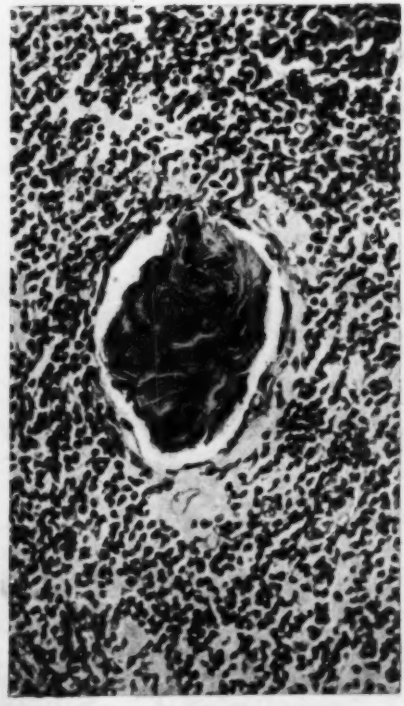

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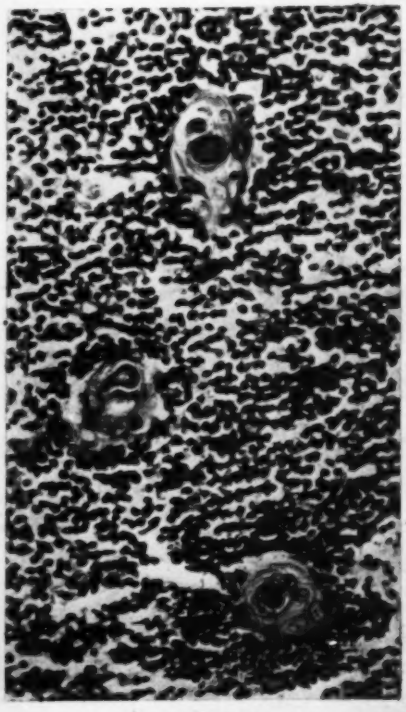

2b

BRANCHIAL TISSUES AS SOURCES OF DIAGNOSTIC ERRORS AT SCALENUS BIOPSIES (DANIELS' BIOPSIES)

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In modern medicine there is an increasing diagnostic activity in order to obtain the pertinent information as early and as complete as possible.

This has led to the use of new diagnostic procedures on one hand, and to the necessity of analysing very early changes on the other.

Through the systematic introduction of new diagnostic routine exaninations one may also encounter unexpected findings, belonging to the realm of aberrations from the normal, without being of any pathological significance, except possibly as causing diagnostic errors.

A routine examination of the "scalenus biopsies" (Daniels') will occasionally reveal cases of aberrant normal tissue, which may be subject to misinterpretations.

The first case encountered was a biopsy with a few very small normal lymph nodes embedded in normal fatty tissue, and in addition a small nodule, the size of a pin-head, consisting of groups of small regular polyhedral epithelial cells forming a mosaic within a fine connnective tissue. Each cell had sharp limitations with a pale, clear cytoplasm and regular round nuclei. The nodule was in another laboratory regarded as a metastasis of a lung adenoma.

In our own material we shortly afterwards had a similar case and our diagnosis was in both cases: "aberrant parathyroid tissue" (Fig. 1).

In the other case in addition to a group of small regular lymph nodes, also several other small nodules were presented, and with the superficial appearance of lymph nodes, but with scattered groups of keratinizing cells in the pulp. The patient was under the suspicion of suffering from "lung cancer", and the immediate reaction was a diagnosis of "squamous cell carcinoma". At a closer study, however, the pattern was not the usual. Firstly, some of the keratinizing cells were very atrophic with pycnotic nuclei and surrounded by keratin scales in concentric layers, giving the picture of Hassal bodies. Secondly, well preserved keratinizing cells were typical squamous cells, and they formed regular small groups, of even size and evenly scattered in the pulp of the nodes. Our diagnosis was "aberrant thymus tissue" (Fig. 2).

Aberrant parathyroid glands and persistent thymus tissue in the vicinity of the scene of development and descent are well known, and may in rare cases have caused some macroscopic surgical problems.

The presence of microscopic remnants are therefore not at all surprising. They may, however, previously not have provoked much interest, if accidentally observed, but to-day it may be useful to have such encounters in mind as possible sources of diagnostic errors.

EXPLANATION OF PLATE

FIG. 1.—Parathyroid tissue.

FIG. 2.—(*a*) Thymus tissue with Hassal body. (*b*) Thymus tissue with regular keratinizing cells.

THE EFFECTS OF X-IRRADIATION ON ADRENAL FUNCTION IN MAN

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THE effects of X-irradiation on adrenal function are not well understood. Increased adrenocortical activity appears to result from irradiation in the experimental animal. This is suggested by indirect measurement of adrenal function (Leblond and Segal, 1942; Lafargue, Schiller, Tyan and Pitoy, 1953; Venters and Painter, 1951; Wexler, Pencharz and Thomas, 1952; Nins and Sutton, 1954; Patt, Swift, Tyree, and John, 1947; Nizet, Heusghem and Herve, 1949), and more directly by measurement of urinary and plasma 17-hydroxycorticosteroids (Brayer, Glasser and Duffy, 1954; Bowers, Nelson, Bay and Samuels, 1952; French, Migeon, Samuels and Bowers, 1955; Van Cauwenberg, Fischer, Viliens and Bacq, 1957). In man, the response to therapeutic irradiation is less well defined. Lasser and Senstrom (1954) and Soanes and Dodson (1954) have found evidence of adrenal hypofunction following X-irradiation of the abdomen, whilst Notter and Gemzel (1956) have reported a rise in plasma hydrocortisone concentration following a transient fall which occurred soon after irradiation. It was thought therefore that a further study of adrenal function in man, as reflected by plasma hydrocortisone concentrations before and after therapeutic X-irradiation, might be of interest.

METHODS

Male and female patients (Table I) undergoing radiation treatment for various malignant disorders were studied. Plasma was obtained on the day before commencement of treatment and on the day after completion of a course of therapy of variable length (12–43 days). In order to eliminate the effect of diurnal variation the blood was drawn at the same hour in both cases (10.00 a.m.). Three patients were studied in greater detail during the five hours after their first X-ray treatment.

Details of therapy received are indicated in Table I. Ten patients were treated on the Linear Accelerator, one received treatment under the Cobalt beam, two received injections of radioactive gold, and the remainder were treated with a 250 kV. therapy machine with 1 mm. Al and 1 mm. Cu filters. The dose varied from 4500 to 6400 rads for patients treated with the linear Accelerator, and from 2000 to 9200 r for those patients treated by other means. The duration of treatment varied from 12 to 43 days.

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Plasma hydrocortisone was determined by the method of Peterson, Kramer and Guerra (1957) with the following modifications:

(*a*) Methylene dichloride was shaken twice with concentrated sulphuric acid, neutralized with sodium hydroxide, washed four or five times with water, and redistilled.

(*b*) Phenylhydrazine was dissolved in ethanol and treated with activated charcoal. It was recrystallized after filtration.

(*c*) The extraction technique was simplified by vigorously shaking the plasma with methylene dichloride for one minute in 8 in. "Quickfit" stoppered tubes. No trouble was experienced with emulsions.

Procedures (*a*) and (*b*) reduced the reagent blank considerably. The plasma was separated immediately following collection and frozen until analysed.

For convenience most analyses were performed on plasmas which had been stored in the frozen state. This made no significant difference in the results of analyses. Ten specimens were analysed before and after freezing for periods up to three weeks and the mean difference in plasma hydrocortisone concentration was $1{\cdot}1 \pm 2{\cdot}0$ μg. per cent.

Plasma hydrocortisone concentrations were measured in a group of in-patients with various non-endocrine disorders, and in a small group of normal healthy males, in order to establish the normal range in non-irradiated subjects, observed over a comparable period of time.

RESULTS

Plasma hydrocortisone concentrations were measured in 33 male and 20 female in-patients with various non-endocrine disorders. The mean for males was $16{\cdot}3 \pm 6{\cdot}1$ μg. per cent. with a range of 6·0–27·4 μg. per cent. For females the mean was $16{\cdot}7 \pm 7{\cdot}7$ μg. per cent. with a range of 3·2–36·0 μg. per cent. The combined group mean was $16{\cdot}5 \pm 6{\cdot}7$ μg. per cent. with a range of 3·2–36·0 μg. per cent. This group includes the hydrocortisone concentrations of patients who were subsequently given therapeutic irradiation, with the exception of those in a terminal state (because of the associated rise in plasma hydrocortisone concentration in such patients (Perkoff, Sandberg, Nelson and Tyler, 1954)).

There was no consistent difference between plasma hydrocortisone concentrations before and after irradiation in 10 male and 10 female patients (Table I). The mean plasma hydrocortisone concentration before radiation was $13{\cdot}4 \pm 8{\cdot}6$ μg. per cent., and after a period of radiation (mean duration 25 days) it was $13{\cdot}8 \pm 6{\cdot}2$ μg. per cent. These figures differ in no significant way from the control group of 10 subjects which gave mean values of $15{\cdot}8 \pm 5{\cdot}7$ μg. per cent. before and $16{\cdot}1 \pm 4{\cdot}7$ μg. per cent. after intervals of similar duration, but without radiation treatment. It is of interest that one of these patients (No. 11) had a normal plasma hydrocortisone concentration at a time when she was suffering from radiation sickness.

The effects of radiation on plasma hydrocortisone concentrations in three subjects, observed over a period of five hours following the first treatment with deep X-ray to the abdomen, is shown in Fig. 1. In two subjects receiving 150 r (50 r through each of three portals), there was a sharp fall in the plasma hydrocortisone concentration, followed by a rise, while in the single patient who received

TABLE I.—*Showing Clinical and Therapeutic Details, and Plasma Hydrocortisone Concentrations Before and After Radiation*

Patient No.	Diagnosis	X-ray therapy received				Plasma hydrocortisone conc. in μg. per cent	
		Type	Site	Duration (days)	Dose (rads)	Before	After
Males							
1	Ca Bladder	L.A.	Pelvis	29	5000	12·6	14·6
2	Ca Lung	,,	Chest	28	4500	10·6	11·3
3	Ca Spine	X-ray	Spine	13	6500	17·3	13·3
4	Ca Bladder	L.A.	Pelvis	29	5500	6·0	6·5
5	Ca Oesophagus	,,	Chest	43	6400	9·3	14·8
6	Lymphoepithelioma	X-ray	Neck	29	5640	9·2	17·3
7	Ca Bronchus	,,	Chest	12	9200	31·0	32·2
8	Ca Larynx	Gold	Neck	26	3480	10·6	14·7
9	Ca Bladder	L.A.	Pelvis	28	5000	7·7	7·4
10	Ca Oesophagus	,,	Chest	25	6205	11·1	6·4
Females							
11	Ca Bowel	L.A.	Abdomen	30	3500	20·6	16·0
12	Ca Uterus	,,	Pelvis	35	4500	5·0	16·3
13	,, ,,	,,	,,	32	4300	21·3	18·4
14	Ca Cervix	X-ray	,,	14	2800	36·0	16·8
15	,, ,,	,,	,,	24	7800	11·8	10·6
16	Metastatic Ca	,,	Neck	22	2500	3·2	17·1
17	Lymphosarcoma	,,	Pelvis	36	2000	16·7	9·0
18	Ca Cervix	Co^{60}	Vulva	30	6100	5·4	4·8
19	,, ,,	L.A.	Pelvis	25	3000	7·7	8·2
20	Ca Ovary	X-ray	,,	30	3670	14·0	20·8

L.A. = Linear accelerator.

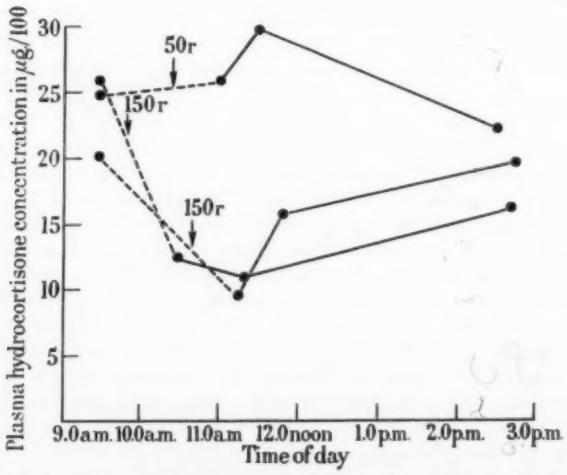

FIG. 1.—Plasma hydrocortisone concentration in μg/100 ml. in three patients before, and during the first five hours following, a single dose of X-irradiation. Two patients received 150 r for carcinoma of the ovary, and the third patient received 50 r for lymphoblastoma with hepatic involvement. Because of the delay between taking the control sample and administration of the radiation, the actual rate of change of plasma hydrocortisone after irradiation is not known. The first and second points are therefore joined by a dotted line.

50 r (25 r through each of two fields) there was a slight rise only in plasma hydrocortisone concentration without an antecedent fall.

DISCUSSION

Peterson, Kramer and Guerra (1957) have reported a normal mean plasma hydrocortisone concentration of 15·0 ± 4·5 μg. per cent. with a range of 6·0–25·0 μg. per cent. for their method of measuring plasma hydrocortisone. The results reported here agree well with these figures and demonstrate the ready applicability of the method. Further we have demonstrated that storage of plasma in the frozen state does not impair the reliability of the technique.

A review of the earlier literature suggests that either adrenal stimulation or depression may occur after X-irradiation according to the site and the dose of the radiation. Whole body irradiation would appear to result in adrenal stimulation, as both urinary (Brayer, Glasser and Duffy, 1954) and plasma (Bowers, Nelson, Bay and Samuels, 1952 ; French, Migeon, Samuels and Bowers, 1955 ; Van Cauwenberg, Fischer, Viliens and Bacq, 1957) corticosteroids have been reported to be increased following irradiation of several animal species. It seems possible therefore that irradiation is another example of a non-specific adrenal stress. However, direct irradiation of the adrenal glands leads to depression of their function. Ungar, Rosenfeld, Dorfman and Pincus (1955) have demonstrated a depression of steroid synthesis following irradiation of the isolated perfused calf adrenal, which would help explain the earlier observation of Edelmann (1951) that shielding of rat adrenals from whole body irradiation increased their survival rate. The early fall in plasma hydrocortisone concentration, observed in two patients exposed to abdominal field irradiation appeared greater than could be attributed to diurnal decline (Peterson, 1957). It is probably explained by a transitory adrenal depression, and is similar to the findings of Notter and Gemzell (1956) who observed a depression in plasma 17-hydroxycorticosteroid concentration 30 minutes after X-irradiation of the adrenal. These workers also noted a rise two to three hours after irradiation. Similarly a rise was observed in two of the three patients presented here, which occurred despite the normal diurnal decline. This rise may have been due to recovery of adrenal function or to temporarily decreased renal excretion of steroids following irradiation. The latter is unlikely because the increase in plasma hydrocortisone concentration is relatively slight even in complete renal shutdown (Englert, Brown, Willardson, Walach and Simons, 1958). On the basis of previous work (Bowers, Nelson, Bay and Samuels, 1952 ; French, Migeon, Samuels and Bowers, 1955 ; Van Cauwenberg, Fischer, Viliens and Bacq, 1957) this rise may indicate adrenal stimulation following X-irradiation.

Failure of the patient who received only 25 r to each of two abdominal fields, to show a fall in plasma hydrocortisone concentration, is in keeping with the observation of Notter and Gemzell (1956) that at least 50 r delivered in a single dose to the adrenal area was necessary to produce a depression of adrenal function. Likewise it has been observed in monkeys that a minimum of 50 r whole body irradiation is required to produce an adrenal response (French, Migeon, Samuels and Bowers, 1955).

The lack of change observed in plasma hydrocortisone concentrations following long periods of radiation therapy, in both our patients and those reported by

Notter and Gemzell (1956) confirm that the change in adrenal function after a single exposure to radiation is transient.

The finding that the plasma hydrocortisone concentration did not change during irradiation sickness indicates that the adrenal is not primarily concerned in this condition (Lasser and Stenstrom, 1954).

It appears that the adrenal response to therapeutic radiation is dependent upon whether the adrenals receive direct irradiation. If the adrenals are not exposed directly to irradiation, the response is similar to that observed following other non-specific stresses : if they are, transient adrenal hypofunction may occur. The practical implication of this and the work of Notter and Gemzell (1956) is that signs of adrenal insufficiency should be watched for when patients receive large doses of irradiation over the abdomen or lower thorax.

SUMMARY

(1) Plasma hydrocortisone concentration was measured in a group of hospitalized patients before and after a course of radiotherapy, and in a comparable group of non-irradiated hospital patients. There was no significant change in the plasma hydrocortisone concentrations after radiotherapy.

(2) In three patients changes in plasma hydrocortisone concentrations were observed over a five hour interval after a single treatment with X-irradiation. In two patients given 150 r the plasma hydrocortisone concentration fell and then rose. In a third patient given only 50 r, there was no initial fall but the plasma hydrocortisone concentration rose slightly.

(3) It is concluded that transient adrenal depression may occur after sufficient irradiation of the adrenal glands.

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THE ROLE OF CONNECTIVE TISSUE PROLIFERATION IN INVASIVE GROWTH OF NORMAL AND MALIGNANT TISSUES: A REVIEW

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ABILITY to invade surrounding tissues is obviously one of the basic characteristics of malignant neoplasms but, in spite of this, the number of investigations dealing with this problem is not very large. Only a few reviews are devoted to this problem, among which the works of Willis (1952), Coman (1946, 1947) and Leighton (1957) should be mentioned. The main aim of this article is to discuss possible mechanisms of invasion and, in particular, to stress the possible role of connective tissue proliferation in this process.

I. Invasive Growth of Non-malignant Tissues

So-called " inflammatory proliferations " of epithelium are probably the best studied examples of a process where invasive growth of the non-malignant epithelium can be observed. In 1906 Fischer described cancer-like epithelial lesions induced in the skin of rabbit ear by subcutaneously injected solutions of scarlet red. These lesions were morphologically similar to squamous carcinomas, but invariably regressed after some period of time. Various opinions have been expressed about factors which might induce such proliferation (see review of the early investigations in this field in books by Parin (1912) and Garschin (1939)). Garschin (1927*a*, *b* ; 1928*a*, *b*, *c* ; 1937 ; 1939) studied in detail the morphology of this process and came to the conclusion that invasive growth of skin epithelium was closely connected both in time and place with the inflammatory changes in subepithelial connective tissue. He supposed that lesions induced by scarlet red in the rabbit skin as well as some types of atypical epithelial proliferations observed in human pathology, belong to the class of processes designated by him as " inflammatory proliferations of epithelium ". Invasive growth of the non-malignant epithelium into the connective tissue at certain stages of inflammation (sometimes with formation of cysts around necrotic areas or around foreign bodies and with subsequent elimination of these bodies through the epithelium-covered surface) and, lastly, complete regression of the epithelial sprouts after the end of the inflammation, are, according to Garschin, characteristics of such proliferations. Inflammatory proliferations of epithelium were induced with aid of different irritative agents in various organs of laboratory mammals ; in lungs (Garschin and Pigalev, 1931*a*, *b* ; Schabad, 1933 ; Garschin and Schabad, 1935, 1936), in kidneys (Zacharievskaja, 1938), in salivary glands (Iskra, 1938), in mammary glands (Golovin, 1952) and in the skin of the rat embryos (Fedorova, 1952). Similar processes of epithelial proliferation and of invasion were observed in the

experiments of Zawarsin and his collaborators, who studied the comparative morphology of experimental inflammatory reactions in invertebrate and lower vertebrate animals, namely, insects (Lazarenko, 1924, 1928), crustacea (Danini, 1925, 1928), molluscs (Zawarsin, 1925, 1927) and frogs, (Braun, 1945). In all these experiments the authors observed invasion of the young connective tissue surrounding the implanted pieces of celloidin by the epithelial sprouts growing from the adjacent epithelial structures; in many cases formation of epithelial cysts around celloidin was observed. Zawarsin (1947) emphasizes that formation of the immature connective tissue under the basal membrane always precedes the beginning of the invasive growth of epithelium during inflammation.

Lazarenko (1935, 1939, 1948) implanted subcutaneously into rabbits and guinea-pigs pieces of celloidin mixed with the minced tissue of various organs of homologous animals (homotransplants of salivary glands, kidneys, thyroid gland and of other organs were used; see also articles of Galustjan (1948), and of Chistovich (1948), where similar experiments respectively with thymus and with pancreas are described). Proliferation of immature fibroblasts around the implanted foreign bodies was observed in these experiments, whereas epithelial cells grew infiltratively from the transplants into this fibroblastic tissue and formed varied organoid structures. Obviously, the interrelationship between the transplanted epithelium and the surrounding connective tissue was essentially the same in these experiments as in the "inflammatory proliferation" described above.

Ectodermal proliferations associated with the mesodermal lesions that can be induced in the chorio-allantoic membrane of the chicken embryos, probably belong to the same group of processes (Olshevskaja and Pogosianz, 1958).

The invasive growth can also be observed during various processes of normal morphogenesis in embryos and in adult animals. For instance, in pregnancy, the mouse mammary gland epithelium forms the alveolar buds which grow into the surrounding connective tissue. Toustanovsky and Vasiliev (1957) have studied the changes in the stroma of mouse mammary gland during pregnancy and lactation; various morphological and histochemical methods were used. It was observed that before the growth of epithelium began, the compact basal membrane disappeared and a net of thin fibres formed around the ducts. Later, the formation of the reticulin fibres around growing alveolar buds proceeded. Development of the young connective tissue, containing the reticulin fibres and the acid mucopolysaccharides which gave metachromatic staining with toluidine blue, were observed also in the endometrium of different mammals around the placental villi growing into the wall of pregnant uterus (Wislocki and Dempsey, 1946, 1948; Davies, 1956). It may be concluded that the invasive growth of the mouse mammary gland epithelium and that of the placental villi is somewhat similar to the "inflammatory proliferation" of epithelium. In all these cases formation of the "bed", consisting of the young connective tissue, precedes the epithelial invasion. The relationships of the connective tissue changes and of the epithelial growth may be different in different processes. For instance, during "inflammatory proliferation" the growth of connective tissue is induced by irritants such as scarlet red or celloidin; the young connective tissue in its turn induces the invasive growth of the adjacent epithelium. It is possible, that in other cases the epithelium, stimulated by some endogenous agent (such as hormones), begins to secrete a substance which induced the proliferation of the connective tissues.

Finally, the proliferation of the epithelium and that of the connective tissue may be under the control of different mutually independent mechanisms; this is probably the case in placental growth. However, in all cases formation of the young connective tissue " matrix " seems to be essential for the invasive growth of the non-malignant epithelium.

II. The Interaction of the Growing Tumour with the Surrounding Connective Tissue

Enzymes, depolymerizing the components of the connective tissue, and tumour invasion

It has been suggested many times that the invasive growth of malignant tumours is a result of the destruction of the intercellular components of connective tissue (fibres or ground substance) caused by some agents released by cancer cells. Bierich (1927) ascribed this action to the lactic acid. When Duran-Reynals had discovered the " spreading factor", which later had been identified as hyaluronidase, many investigators made efforts to find some evidence in favour of the view that an agent of the same type was responsible for invasiveness of malignant neoplasms. Several authors have described stimulation of growth and increase of invasiveness of different transplantable tumours (Gopal-Ayengar and Simpson, 1947; Simpson, 1950; Balitzky, 1950; Podilchak, 1951; Kraul, 1955) and acceleration of the development of spontaneous mammary tumours in mice (Lacassagne, Loiseleur and Rudali, 1957*a*, *b*) after injections of the testicular extracts or of purified hyalyronidase preparations. However, many other investigators (Tanzer, 1932; Prime and Haagensen, 1934; Coman *et al.*, 1947; Arnesen, Buxton and Dulaney, 1949; Seifter and Warren, 1950; Lührs and Willig, 1952) did not observe any influence of hyaluronidase on the growth of tumour grafts or even describe inhibitory effect.

The efforts to find " spreading factors " in the tumour tissue also were not very successful in the majority of the experiments. Boyland and McClean (1935) found the " spreading factor " in aqueous extracts from tissue of rapidly growing transplantable tumours; activity of this factor was approximately proportional to the rate of growth of the neoplasm. Pirie (1942) observed weak hyaluronidase activity in 5 transplantable animal tumours. However, at the same time Gibertini (1942) did not find hyaluronidase in the majority of the extracts of 23 human tumours. McCutcheon and Coman (1947) came to the conclusion, that hyaluronidase is present only in a part of human neoplasms; activity of the enzyme in these tumours is weak. Dux, Guerin and Lacour (1948) studied 17 human tumours and 19 experimental neoplasms of animals and did not find any correlation between the malignancy of cancer and the presence of hyaluronidase. In the experiments of Gluzman (1950) " spreading factor " was found in the extracts from different grafted and methylcholanthrene-induced animal tumours. According to Podilchak and Petrus (1952) rapidly growing malignant human neoplasms contain hyaluronidase, whereas in benign tumours this enzyme is not present. However, Kiriluk, Kremen and Glick (1950) suggest that not the malignant cells themselves, but micro-organisms, contaminating neoplasm, are the source of hyaluronidase in the tumour tissue and, in fact, these authors did not find any hyaluronidase activity in tumours when special measures were taken to avoid bacterial contamination. Similar results are reported by Reggianini (1953).

Balasz and von Euler (1952) came to the conclusion that concentration of hyaluronidase is higher in necrotic parts of the Walker tumour than in the living tissue of the same tumour.

As one may see from this list of controversial findings, the opinion that hyaluronidase is the factor requisite in all cases for invasive growth is in all probability unfounded.

Some investigators think proteolytic enzymes liberated by cancer cells and not hyaluronidase play an important role in invasion. Gersh and Catchpole (1949) describe depolymerization of the PAS-positive ground substance of connective tissue by a rapidly growing transplanted tumour ; they suggest, that this depolymerization is due to the action of collagenase-like enzymes. Similar hypothesis has been put foward by Sylven (1949), who observed dissolution of the connective tissue fibres around human carcinomas. Sylven and Malmgren (1955) found that the proteolytic activity was greater in the peripheral part of the nodule of grafted tumour than in the central part of the same nodule. It is not clear, however, whether these proteolytic enzymes act *in vivo* inside the tumour cell or are liberated in the surrounding tissue.

Experiments with tissue cultures do not confirm the existence of a positive correlation between the proteolytic activity and the invasiveness of neoplasms. As the *in vitro* investigations of Santesson (1935) and later those of Leighton (1957) showed, the most malignant tumours are least proteolytic, and conversely the least malignant tumours are the most proteolytic. Leighton (1957) suggests that the capacity to make the ground substance more fluid is accompanied by a limitation of invasive spread.

We see that at present there are no facts that unequivocally confirm the view that hyaluronidase, proteases or other depolymerizing enzymes play a leading role in the invasive growth of tumours. On the contrary, some of the available data are inconsistent with such hypotheses.

Interaction of tumour explants with connective tissue in vitro

Some observations show that during invasive growth a complex interaction of the tumour and of the surrounding tissue takes place and that the proliferation of connective tissue is probably an important part of such interaction.

In the experiments by Leighton (Leighton and Kline, 1954 ; Leighton *et al.*, 1956 ; Leighton, 1957) the interaction of malignant human cells (HeLa carcinoma, D–189 line) and of normal tissues was studied *in vitro*, in sponge matrix tissue cultures. HeLa cells readily invaded those normal tissues of the chick embryo and of the human foetus that gave rise to a luxuriant outgrowth of connective tissue. It was shown also that malignant cells of the D–189 line and explants of normal connective tissue are attracted by, and move toward, one another. When the contact between these two tissues is established, malignant cells begin to invade connective tissue. Stimulation of the growth of connective tissue by tumour explants was observed also in the experiments of Fischer, Laser and Meyer (1929), of Santesson (1935) and of Ludford and Barlow (1944).

Wolff and Wolff (1958) successfully cultivated human malignant cells (KB strain) together with pieces of mesonephros of chick embryos. It would be important to find out whether the mesenchymal tissue of chicken embryo is the component responsible for stimulation of tumour growth in such combined cultures. It is interesting to note in this connection that as Schleich (1956) showed, the

presence of normal connective tissue is necessary for survival of malignant cells of the Yoshida sarcoma *in vitro*. Powell (1957, 1958) suggests that monocytic cells contained in the explanted pieces of normal embryonic organs form *in vitro* some substances which are essential for growth of cells of the Ehrlich mouse ascites carcinoma.

Inflammation and invasive growth of transplanted tumour cells

Among the *in vivo* experiments confirming the important role of connective tissue reactions in the invasive spread of cancer cells, the investigations dealing with influence of inflammation on the growth of tumour transplants need discussion.*

It was shown (Devic *et al.*, 1950 ; Vasiliev, 1955) that malignant cells migrate from subcutaneous grafts of mouse and rat tumours into undifferentiated connective tissue developing around such grafts after the initial inflammatory reaction. Inflammatory reactions around transplants of heterologous tumours were not found to be different in any detail from similar initial reactions around transplants of isologous and homologous neoplasms. Malignant cells of heterografts also migrate into the young connective tissue and begin to multiply there (Vasiliev, 1958*a*). The suggestion has been put forward therefore that this connective tissue proliferation is favourable for initial spread of malignant cells from grafted tumour fragments (Devic, *et al.*, 1950 ; Vasiliev, 1955, 1958*a*). It is probable that suppression of the initial inflammation and of the following connective tissue proliferation is one of the main causes of the inhibitory action of cortisone on the growth of transplanted homologous tumours which has been observed by a number of authors (Heilman and Kendall, 1944 ; Antopol, Glaubach and Graff, 1954 ; Higgins and Bennet, 1952 ; Martinez and Bittner, 1955 ; Selye, 1955 ; Vasiliev, 1958*a*). Inhibition of the invasion of the connective tissue by carcinomatous cells from tumour fragment grafted to cortisone-treated mice have been confirmed by histological examination (Antopol, Glaubach and Graff, 1954 ; Vasiliev, 1958*a*).

It is obviously important to find out whether the stimulation of inflammatory reaction and of connective tissue proliferation may have an opposite effect, i.e. to enhance the spread of malignant cells and to increase the rate of growth of transplanted tumours. Experiments in which the influence of artificially increased inflammation on the growth of transplanted tumours were studied gave rather contradictory results : some authors (Kubo, 1930 ; Chambers and Grand, 1937 ; Pigarevsky, 1952) observed inhibition of tumour growth ; a few investigators (Molomut *et al.*, 1955 ; Hewett, 1956) did not find any effect at all, while a number of authors (see below) report that increased inflammation enhances the growth of grafts. Such contradictions may be due to differences in methods used for stimulation of inflammation and to differences in the morphology of inflammation induced by these methods. It is possible, for instance, that rapid elimination of such irritants as formic acid or allergic protein from the site of injection may account for the absence of any effect of inflammation reported by Molomut *et al.* (1955) and by Hewett (1956). Inhibition of tumour growth by infusorial earth

* It is to be stressed that only the role of initial inflammatory reactions around tumour grafts will be discussed here. Investigations dealing with other types of reactions of connective tissue cells, for instance, reactions associated with rejection of foreign tumour grafts, are obviously beyond the scope of this article.

(Kieselguhr) observed in experiments of Kubo (1930) and of Pigarevsky (1952) may be a result of the direct toxic action of this irritant on the transplanted tumour cells (see Vasiliev, 1957). Inflammation associated with extensive suppuration and tissue necrosis obviously may have a harmful effect on the tumour cells. However, it is important for our discussion that the majority of authors come to the conclusion that productive inflammation associated with proliferation of the connective tissue cells favours the establishment and growth of tumour transplants and the spread and metastasis of malignant cells.

Tsanev and Marcow (1956) in experiments with transplantable Guerin carcinoma observed that grafting of the tumour into a 6–9-day-old granulation tissue increased the frequency of metastasic growths in regional lymph glands and also shortened the average latent period of their appearance and the survival time of tumour-bearing rats. According to Podilchak (1955) Brown-Pearce rabbit carcinoma metastasized more often to the spleen if a focus of chronic inflammation had been induced in that organ. In experiments described in another communication, Podilchak (1956) observed an increase of the frequency of metastasis of the Brown-Pearce tumour to the stomach after the induction of chronic inflammation in the organ. Zahl and Novac (1949) reported that mechanical injury of the transplantation site increased the rate of growth of transplantable mouse sarcoma. In the experiments of Jones (1926) transplantable mouse mammary gland tumour of DBA strain could be transplanted successfully to otherwise resistant C57Bl mice if a local irritant (a piece of sterile flannel) was introduced subcutaneously together with the tumour graft.

In a number of recent communications a new technique for study of inflammation—so-called " air-pouch technique " described by Selye and Horava (1952) has been used. In the experiments of Selye (1955) Walker carcinosarcoma was grafted into the " air pouches " of rats ; combined treatment of animals with small doses of cortisol and with NH_4Cl, which suppressed accumulation of inflammatory exudate in adrenalectomized rats also inhibited the growth of the transplanted tumour. Robert (1954) reported that grafting of transplantable mouse mammary tumour into an " air-pouch " made on the neck of a mouse resulted in stimulation of growth of the graft as compared with routine subcutaneous transplantation. On the contrary, Hewett (1956) did not find any difference between the growth rates of mouse Sarcoma 37 transplanted subcutaneously and in an " air-pouch ". It is possible, however, that in these experiments the quantity of the tumour tissue injected to each animal was too small and did not induce a sufficient degree of inflammation in the " air-pouch ". This suggestion is confirmed by the fact that the accumulation of exudate in the " air-pouches " with grafted tumours, which had been observed by other investigators, did not occur in Hewett's experiments.

Vasiliev (1957) observed that after injection of tumour suspension in an " air-pouch " the initial inflammation was more pronounced and the young connective tissue formed after such inflammation covered a much wider area than after subcutaneous grafting. Striking stimulation of growth of a number of isologous and strain-non-specific homologous grafts of mouse and rat tumours transplanted into " air-pouches " was observed in these experiments. Mouse Sarcoma 180 grafted into " air-pouches " of weanling rats grew there much more rapidly than when transplanted subcutaneously, so that the majority of animals died with huge tumours in " air-pouches " 6–8 days after grafting ; it was possible to propagate

mouse Sarcoma 180 serially in the " air-pouches " of weanling rats in a number of passages without cortisone treatment of the heterologous hosts. If animals did not die from tumour growth in the first 10 days of the experiment, heterotransplants in the " air-pouches " regressed at the same time as in control animals with subcutaneous grafts of the same mouse tumour : that is from 11 to 13 days after transplantation. Other mouse tumours used in these experiments (RSM strain of mammary gland carcinoma and hepatoma XXII) regressed in untreated weanling rats earlier than Sarcoma 180—at 7–8 days after grafting. In cortisone-treated rats these tumours grew for a long period ; the final weight of these heterologous tumours transplanted into " air-pouches " was 3–4 times as high as in control rats with subcutaneous transplants, which received similar cortisone treatment.

Selye (1957) grafted suspension of the Walker tumour into " air-pouches " made on the backs of rats ; simultaneously croton oil was injected into " air-pouches " of some of the rats. Selye came to the conclusion that the stimulation of inflammation by croton oil enhances the growth of transplants.

Results of the experiments quoted above show that stimulation of inflammation and of connective tissue proliferation around grafts of homologous and heterologous tumours can in many cases facilitate the establishment and enhance the growth of such grafts. These data are in good agreement with the idea of the importance of connective tissue proliferation for invasive growth of malignant cells.

Influence of embryonic tissue on the growth of tumour transplants

Experiments of another type, which deserve discussion here, are those dealing with the effect of embryonic tissue on the growth of tumour transplants. Greene (1949, 1955) reported that in experiments with transplantation of animal and human tumours into the anterior eye chamber or brain of heterologous hosts the percentage of the positive takes increased if pieces of embryonic tissue were added to the graft. Greene suggested that embryonic tissue either evoked stromal reactions of the host or served as primary stroma for the transplanted tumour. As Schneyer (1955) showed, the growth rate of a transplantable mouse mammary carcinoma significantly increased if the tumour suspension had been mixed with isologous embryonic tissue before grafting.

In the experiments of Vasiliev (1958*b*) and of Vasiliev and Olshevskaja (1958) suspensions of embryonic tissues were mixed *in vitro* with the suspensions of tumour cells immediately before transplantation. It was shown that such embryonic tissue suspensions significantly accelerated the growth of homografts of strain-non-specific tumours (mouse Sarcoma 180 and rat Sarcoma 45) and also that of heterografts of mouse Sarcoma 180 in weanling rats. The embryonic tissues, whose species specificity corresponded to that of the host, were the most active, for instance, mouse embryonic tissues were active in experiments with homografts of mouse Sarcoma 180 and rat embryonic tissues in experiments with the same tumour grafted into rats. Embryonic tissue lost its activity after heating to 60° C. or freezing to −40° C. Different homologous and heterologous tissues of adult animals had no growth-accelerating properties. It was suggested that stimulation of tumour growth is due to the presence of living embryonic cells at the site of transplantation. Inactivity of heterologous embryonic tissues may then be regarded as a result of their rapid destruction in the host's organism.

Accumulation of a large number of immature fibroblasts at the site of the injection of suspensions of homologous embryonic tissues had been observed in rats by Vasiliev and Olshevskaja (1958). It is not clear at present whether these cells migrate from the grafted pieces of embryonic organs or are host elements which begin to multiply under the influence of the transplant. It is tempting to suggest that the presence of such fibroblasts is the factor responsible for stimulation of growth of the transplanted tumour. In any case it is obvious that combined transplants consisting of the embryonic and malignant tissue are similar in many respects to combined explants studied in the experiments of Leighton, Wolff and others (see above). In both cases embryonic tissue probably may serve as an artificially provided " matrix " for tumour cells, as an environment favourable for the multiplication and invasive spread of these cells. Thus, the role of embryonic tissue in these experiments is, probably, the same as that of undifferentiated connective tissue developing around a growing tumour.

DISCUSSION

Several suggestions may be put forward on the basis of the facts reviewed above. It seems probably that in many cases proliferation of connective tissue plays an important part in invasive growth of tumours. Possibly such proliferation is not requisite for all processes of invasion ; for instance, it is probably unnecessary for invasion of tissues by white blood cells as well as by their malignant counterparts. However, for many types of tumours the formation of undifferentiated connective tissue seems to be an essential part of the mechanism of invasion. Such connective tissue can have several functions during invasion. It can form a network of thin microscopic and submicroscopic fibres serving as matrix which gives mechanical support for tumour cells. During " inflammatory proliferation " growing connective tissue somehow attracts the adjacent epithelium. It may be suggested that some hypothetical substances liberated by young connective tissue surrounding a tumour also attract the malignant cells which then begin to invade this tissue ; *in vitro* observations by Leighton (see above) give some support to this idea. Finally, young connective tissue can create a uniform chemical environment favourable for tumour cells. We have mentioned already experiments which show that *in vitro* normal tissue explants liberate into the medium some substances essential for survival of the ascites tumour cells. It is possible that connective tissue can form such substances *in vivo*.

Close association of invasive spread and of connective tissue proliferation is not only a characteristic of malignant tumours ; as we tried to show in the first part of this review, similar relationships can be observed during invasive growth of normal tissues. Investigations by Zawarsin and his collaborators make clear that invasive growth of non-malignant epithelium into young connective tissue may occur in invertebrate animals. Thus, such type of morphogenetic reaction has been developed at a relatively early stage of the evolutionary process. The basic mechanisms of invasion are probably the same for normal cells and for malignant neoplasms, but in cancer tissue these mechanisms are at work for indefinitely long periods whereas in normal tissues they start and cease work at a definite time, for instance, at a certain stage of inflammation or under the influence of some endocrine change. It is possible, therefore, that cancer cells acquire an intrinsic ability to evoke proliferation of connective tissue, whereas

normal tissues exhibit this ability only temporarily under the action of factors outside the cell. Invasive properties can develop at different stages of " tumour progression " and are to some degree independent from morphological anaplasia (Foulds, 1954, 1958 ; Hamperl, 1957). For instance, organoid tumours of mammary gland, which are made up of differentiated tubules and end bulbs of glandular epithelium are the most invasive of all mammary gland neoplasms in mice (Foulds, 1956). Some part of the young connective tissue invaded by tumour cells is transformed eventually into a stroma for these cells. Therefore, the ability to induce proliferation of connective tissue favourable for invasion is similar in many respects to the stromatogenic properties of tumours. We should recall here the important investigations of Greene (1951), who suggests that stroma-inducing ability of tumours, which develops at some stage of cancerization, may account for the transplantability of these neoplasms into the brain or anterior eye chamber of heterologous hosts.

The ability to induce adequate proliferation of connective tissue is probably not the only factor which determines invasive properties of tumours. Such factors as increased hydrostatic pressure in the tissue (Young, Lumsden and Stalker, 1950 ; Lumsden, 1957) or changes of the cell surface and of intercellular cement substance (Coman and Anderson, 1955 ; Ambrose, James and Lowick, 1956 ; Cowdry, 1953) may also play an important part in the interaction of tumour cells with surrounding tissues. Absence of " contact inhibition " between normal and malignant cells in tissue cultures (Abercrombie and Heaysman, 1954) is in all probability one of the results of surface changes.

The morphology of the young connective tissue surrounding invading tumours may vary in different neoplasms. Such tissue may or may not contain a network of thin reticulin fibres and acid mucopolysaccharides which give metachromatic staining with toluidine blue (see Sylven, 1949). The layer of growing connective tissue may be very thin and sometimes very difficult to see in histological sections. However, even in such cases proliferation of immature fibroblasts can be easily observed in spread preparations taken near the tumour ; the cytoplasm of these fibroblasts was found to contain large numbers of ribonucleoprotein granules (revealed by fluorochrome acridine orange) and also PAS-positive polysaccharide (Vasiliev, 1958*c*). If the young connective tissue around a neoplasm is not invaded by tumour elements it can probably become collagenized and then it forms the capsule of the tumour nodule, and so to some extent counteracts the spread of malignant cells. It is important to keep in mind that young connective tissue, in which the synthesis of intercellular components is not yet completed, may resemble histochemically connective tissue in the state of depolymerization (Wasserman, 1956). For instance, in both cases polysaccharides of the ground substance may be more soluble in water than those in the normal connective tissue (compare Gersh and Catchpole, 1949).

The mechanism of destruction of normal tissue during tumour invasion is one of the problems which urgently needs further investigations. Experiments discussed in the preceding part of this article do not confirm the view that normal connective tissue is enzymatically lysed by malignant cells but such a possibility cannot be completely excluded at present. Even if it were shown that some of the preparations containing depolymerizing enzymes can stimulate the tumour growth, the possibility of an indirect action of these preparations *in vivo* should be taken into consideration. It is known, for instance, that proliferation of con-

nective tissue cells may be observed after injection of testicular hyaluronidase (Bensley, 1950; Williams, 1955).

Disappearance of normal tissue in an area invaded by tumour can be a result of competition between normal and malignant cells for various substances essential for their metabolism. It has been shown, for instance, that synthesis of desoxyribonucleic acid in isolated nuclei of tumour cells begins at a lower concentration of substrate (mixture of ribonucleotides) than the same process in nuclei of normal cells (Belousova, 1955). It is probable, therefore, that non-malignant cells may die from "starvation" in the presence of tumour elements which use nutritional substances from the environment more efficiently (see also Larionov, 1958). Certain structures of pre-existing tissue such as collagen fibres, basal membranes, etc., may be destroyed not by the malignant cells themselves, but by young connective tissue whose growth is induced by the tumour. In his review on "Intercellular components of connective tissue" Wasserman (1956) stresses that "... fibrolysis is a physiological process occurring in conjunction with growth and adaptive re-organization of connective tissue structures ... Connective tissue cells are likely to play an active part in the process".

Little can be said at present about the possible nature of the factors which induce proliferation of connective tissue around tumours. Probably the action of these factors is not restricted to connective tissue only: agents which induce "collateral hyperplasia" (see review of Foulds, 1940), or proliferation of embryonic tissue grafted in the anterior chamber of mice together with a piece of homologous tumour (Browning, 1952) as well as substances from mouse Sarcoma 180 which stimulate the growth of nerve fibres *in vitro* (Hamburger, 1954; Cohen, Leui-Montalcini and Hamburger, 1954; Levi-Montalcini, Meyer and Hamburger, 1954) may be of the same nature. Recently published data of Cohen and Levi-Montalcini (1957) indicate that nerve growth-promoting factor in Sarcoma 180 is a protein or is bound to protein.

SUMMARY

An analysis of different types of invasive growth of normal epithelium shows that proliferation of underlying connective tissue is in all probability essential for invasion. It is suggested on the basis of varied experimental data that formation of young connective tissue around neoplasms may be important in many cases for invasive growth of malignant cells. The possible role of connective tissue proliferation and mechanisms of its development are briefly discussed.

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THE SIGNIFICANCE OF THE CELL TYPE IN THE FLUORESCEIN-GLOBULIN STAINING OF TISSUES

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THE differentiation of normal epithelial cells from their malignant counterparts in both man and animals has been studied extensively by means of fluorescein-globulin stains (Louis, 1957*b*, 1957*c*, 1958*b*, 1958*c*). The investigation has been extended to include normal and leukaemic blood cells (Louis, 1957*d*) and in animals (Louis, 1958*a*).

It has been shown that the globulin fraction in the stain reacts with a basic protein complex present in normal cells (Hughes, 1958*a*). Its localization within the cell is visualized (in ultra-violet light) by the fluorescing component of the stain which is commonly fluorescein but may be other (and related) dyes. The globulin does not become attached to malignant cells probably because these cells lack the basic protein complex which is found in normal cells (Sorof and Cohen, 1951).

This phenomenon has now been demonstrated in a large number of cases and the consistency of the observations seems to suggest a definite pathological basis at the biochemical level. Several hundred specimens of normal tissues of all kinds have stained and nearly 300 malignant tumours from animals and man have failed to stain. It is the purpose of this paper to discuss the general question briefly and to consider the few cases which appear, at first sight, to be exceptions to the general rule.

Weiler (1952, 1956*a*, 1956*b*) attributed this staining reaction (in the case of rat liver and rat hepatoma) to " organ specificity " and placed the emphasis on the importance and possible significance of antibody-antigen reactions. He suggested that this difference might allow a distinction to be made serologically between normal and neoplastic cells. In the histological part of his work, he had used a γ-globulin which had been obtained from rabbits previously injected with rat liver homogenate. In repeating Weiler's work, Hughes (1957) and Hughes, Louis, Dineen and Spector (1957) were able to reproduce identical differential staining by using any rabbit globulin fraction irrespective of whether or not the donor rabbit has been previously injected with homogenate of rat liver. It was also found that similar observations could be made using globulins of different species including those of the same animal (King, Hughes and Louis, 1958*a*). Furthermore, similar staining reactions could be obtained with the albumins and α-globulins (King, Hughes and Louis, 1958*b*).

These and further studies have emphasized that the reaction was merely a physicochemical one, that is, a protein-protein interaction between the basic cytoplasmic proteins of the tissue cells and the fluorescein conjugated proteins of the stain (Hughes, 1958*a*). Since the conjugation process utilizes free amino groups of the original protein, the fluorescein-protein conjugates would be expected to be

much more acidic than the parent protein. Electrophoretic studies of fluorescein globulin conjugates have indicated that the fraction concerned with immunologically non-specific staining is more highly conjugated (and hence more acidic) than a fraction concerned with immunologically specific staining.

Whether a given tissue stains or not depends upon the presence and amount of substances with an affinity for the stain. So far, all studies have shown that most cells of epithelial nature have an affinity for fluorescein-protein complexes whereas their malignant counterparts do not. In addition to malignant cells, most kinds of connective tissue cells (in the " fixed " and resting forms) as well as red blood cells commonly fail to stain. It is of interest to examine these cells to see if, from a morphological viewpoint, this lack of staining can be related to diminished cytoplasmic bulk or whether it reflects some similarity of the cytoplasmic constituents of malignant and connective tissue cells.

Observations relating to the significance of the staining reaction, although not yet fully understood, are discussed in this paper.

METHODS AND MATERIALS

The methods of study used have been described in detail elsewhere (Louis, 1957*a*, 1957*d*, 1958*a*). Briefly the stains employed have been mainly fluorescein-protein complexes.

The preparation of serum protein fractions from a large series of animals has already been described (King, Hughes and Louis, 1958*a*) and these were conjugated with fluorescein and rhodamine isocyanates by the method of Coons and Kaplan (1950).

Further technical details concerning cutting of unfixed frozen sections, preparation of blood smears, staining, fluorescence microscopy and photography have been published previously (Louis, 1957*a*, 1957*d*, 1958*a*).

As with all previous experiments the routine adopted in the investigation was to photograph representative areas first with ultra-violet light, then the sections or smears were fixed in 10 per cent formol-saline, stained with haematoxylin and eosin and finally, for comparison, photographed again with visible light. In this way identical cells were examined after both staining methods.

RESULTS

The staining characteristics of different types of normal tissues (and tumours arising in them) were investigated by fluorescence microscopy and have been already recorded. These observations included carcinoma of the bowel (Louis, 1957*c*), tumours of the breast (Louis, 1958*b*), tumours of the epidermis (Louis, 1958*c*), human leukaemias (Louis, 1957*d*) and animal leukaemias (Louis, 1958*a*); in addition a large number of normal and tumour tissues using conjugated globulins taken from a series of animals (King, Hughes and Louis, 1958*a*), as well as other serum fractions such as albumins and α- and β-globulins (King, Hughes and Louis, 1958*b*), have been studied.

All the conjugated sera gave identical staining results. Repeated observations showed that only the cytoplasm of innocent epithelial cells and of white blood cells had an affinity for the conjugated dyes and emitted a bright green fluorescence in ultra-violet light. The nucleus of these cells, any additional cytoplasmic

inclusions such as vacuoles or melanin and the minimal amount of intercellular cement supporting the parenchymal cells lacked this affinity and appeared as shadows in the black background. In addition, the connective tissue cells and their intercellular substance, although present in large amounts, also failed to show a positive staining reaction. When connective tissue cells enlarged, with the development of a moderate or considerable amount of protoplasm, they then stained. Examples of these were phagocytic wandering cells, proliferating fixed cells (especially giant cells) and enlarged endothelial or endothelioid cells. In all these cases nuclei failed to stain. However, in such species as the domestic fowl, frog, lizard, axolotl and fish etc., whose erythrocytes possess a nucleus, it was observed that these nuclei had an affinity for the fluorescent dye. Tissues of the central nervous system, within the limits of a few observations on man, rabbit, rat and fowl, have uniformly failed to stain in any way.

Thus of all the general somatic (as opposed to nervous) cell types examined throughout these investigations only the nucleated red blood cells failed to conform to the normal pattern but showed a reversal of the staining reaction, namely, the nucleus did but the cytoplasm did not fluoresce.

The array of non-neoplastic tissues examined is shown in Table I and the neoplastic tissues in Table II. Some examples of these cases are illustrated in Fig. 1–14. The, as yet, incomplete results on the nervous system are not tabulated.

DISCUSSION

If a stain composed of a fluorescein isocyanate-protein conjugate will stain normal tissues in most parts of the body but fails to stain the cells of malignant tumours of a related cell type, the more complete this correlation, the more valuable will the method be, not only from the basic biological point of view but also from the practicability of a staining method to distinguish malignant tumours from other forms of tissue proliferation. Even a casual examination of material, however, will show, in fact, that not all normal tissues stain.

As far as observations have been made to date, it can be said that all parenchymal cells in all the vertebrates and even in some of the invertebrates stain. While the parenchymal cells stain and fluoresce brightly, usually the supporting tissues do not, but in so far as there are some exceptions to this the matter requires further scrutiny. It is necessary, however, in the first place to consider the relation of parenchymal tissues to supporting tissues.

Various types of divisions of tissues have been made, but in general, there are the two main kinds. These divisions may be referred to either as ectoderm and mesoderm (endoderm being comparable with the ectoderm in position) in an early stage of development, or rind and pulp tissues as used by Adami in his classification of tumours or as epithelium and connective tissue.

There has been a certain amount of confusion because of the many attempts to correlate rigidly adult structures with those found at an early stage in the development of the embryo. Thus epithelium has been equated with ectoderm and endoderm and connective tissue with mesoderm. At the same time it has become quite clear that some structures such as kidney and adrenal gland and probably liver are mesodermal in origin, and yet structurally their parenchymal components conform with the general idea of " epithelial " cells. It is for this reason that the term " parenchyma " was used earlier.

TABLE I.—*Results Obtained After Staining Different Normal and Non-malignant Tissues with Fluorescein-protein Complexes*

Comparable results were found in all cases namely, the cytoplasm of all epithelial cells fluoresced well. The number of observations made is greatly in excess of this total since many tissues were examined by a battery of protein conjugates.

Tissue	Condition	Number of cases
Epidermis	(*a*) Normal epidermis (man, mouse, rat, rabbit)	62
	(*b*) Hyperkeratotic wart (man)	8
	(*c*) Molluscum pseudocarcinomatosum (man)	12
	(*d*) Irritated epithelium (man)	12
	(*e*) Sudoriferous adenoma (man)	1
Breast	(*a*) Lactating breast (mouse)	4
	(*b*) Hyperplasia (man)	62
	(*c*) Fibroadenoma (man)	14
Stomach	(*a*) Normal mucosa (man)	22
Colon	(*a*) Normal mucosa (man, mouse, fowl, rat, lizard, frog, sheep axolotl)	50
	(*b*) Polyps (man)	6
Lung	(*a*) Bronchial mucosa (man, mouse)	20
	(*b*) Bronchial adenoma (man)	1
Thyroid gland	(*a*) Normal thyroid (man)	38
	(*b*) Thyrotoxicosis (man)	22
	(*c*) Colloid goitre (man)	26
	(*d*) Foetal adenoma (man)	15
Lymph node	(*a*) Normal (man, mouse, rabbit)	28
Liver	(*a*) Normal (man, mouse, rat, rabbit, axolotl, frog, fowl, sheep)	55
Kidney	(*a*) Normal (man, mouse, rabbit, rat, fowl)	16
Blood	(*a*) W.B.C. (man)	197
	(*b*) W.B.C. (mouse)	160
	(*c*) W.B.C. (fowl, ox, sheep, frog, axolotl)	15
	(*d*) R.B.C. nuclei (fowl, frog, axolotl, fish, lizard)	12
	(*e*) Chronic leukaemia (man)	166
	(*f*) Chronic leukaemia (mouse)	4
Endometrium and cervix	(*a*) Hyperplasia (man)	6
	(*b*) Endometriosis (man)	4
	(*c*) Cervical erosion (man)	7
	Total	1045

Nevertheless the distinction in adult tissues is commonly accepted as being between epithelium and connective tissue. Usually epithelium is composed of cells with a considerable amount of cytoplasm surrounding a well-defined nucleus and the individual cells are arranged in groups with a very small amount of intercellular cement or material. Viewed from this point of view it is of little moment what was the remote origin of the cells. Connective tissues on the other hand are characterized, from the very nature of their function, by a considerable amount of intercellular material and the cell itself is often either small or may be difficult to recognize, the position of the cell being indicated by the nucleus.

The observations therefore, in the employment of the fluorescein-globulin stain, are that epithelial cells in normal circumstances stain well, but connective tissue cells usually fail to do so. Occasionally groups of connective tissue cells do stain and, in such cases, these stand out clearly from the remainder of the connective tissue just as epithelial cells do.

TABLE II.—*Results Obtained by Staining Various Neoplastic Tissues with Different Fluorescein-protein Complexes*

In all cases examined identical differential staining was obtained—normal tissue fluoresced but the malignant counterparts failed to do so.

Tissue	Condition	Number of cases
Epidermis	(*a*) Squamous cell carcinoma (man)	15
	(*b*) Basal cell carcinoma (man)	4
	(*c*) Induced carcinoma (mouse)	6
Breast	(*a*) Carcinoma (man)	34
	(*b*) Spontaneous carcinoma (mouse)	18
	(*c*) Transplanted carcinoma (mouse)	14
	(*d*) Walker 256 carcinoma (rat)	7
Stomach	(*a*) Carcinoma (liver)	13
Colon	(*a*) Carcinoma (man)	28
Lung	(*a*) Carcinoma (man)	9
Thyroid	(*a*) Carcinoma (man)	3
Lymph node	(*a*) Secondary Melanoma (man)	3
	(*b*) Secondary carcinoma (man)	7
Liver	(*a*) Hepatoma (man)	3
	(*b*) Cholangioma (man)	1
	(*c*) Hepatoma (rat)	18
	(*d*) Cholangioma (rat)	8
	(*e*) Secondary carcinoma (ox)	1
	(*f*) Secondary carcinoma (pig)	1
Kidney	(*a*) Carcinoma (man)	3
Adrenal	(*a*) Carcinoma (ox)	1
Blood	(*a*) Acute leukaemia (man)	19
	(*b*) Acute leukaemia (mouse)	60
	(*c*) Leucosis (fowl)	1
	(*d*) Leukaemia (sheep)	3
	(*e*) Leukaemia (ox)	1
	Total	281

In this differentiation of epithelium from ordinary connective tissue the same kind of technical approach as is employed with all types of stain must be adopted. There is an optimum time for staining in order to provide the most satisfactory delineation of the various structures being examined. Obviously if the stain is applied for too short a time tissues will not stain. On the other hand if the stain is allowed to act for too long a period, overstaining of the tissue occurs and even those which do not ordinarily take up some of the stain may do so ; thus a degree of differentiation which will allow the distinction of the various parts cannot be obtained. To this extent times of staining and concentration of stains must be arbitrary, but it is no more arbitrary than is the case with every other stain that is employed in histological work.

The essential component of the stain used is the protein ; since this is not ordinarily demonstrable visually it is conjugated with a dye. The concentration of material obtained in a cell is so small that most dyes employable in this way are not sufficiently easily recognizable to make the observations easy, rapid and certain. It is for this reason that the fluorescing dyes have been used—and this adds some further complications.

The observations are made on the effect of incident ultra-violet light so that a fluorescence is observed. The colour of this fluorescence depends on the dye

used and in the one most commonly used—fluorescein—the fluorescence is of a green colour ; when rhodamine is used the result is orange—and still others may be employed. Some of the tissues in the body have a natural fluorescence (in ultra-violet light) which is independent of the staining, and although this may be prominent, because of the difference in colour of the fluorescence it should not present any significant problem ; it is important that this auto-fluorescence should be thoroughly appreciated. Not only does the difference in colour of the fluorescence light indicate the nature of this phenomenon, but the distribution of the fluorescing material clearly indicates that, in its morphological arrangement, it is different and distinct from the cells of the tissue.

The important connective tissue component which fluoresces naturally is elastic tissue. This has a linear distribution and occurs characteristically in some situation such as the walls of small blood vessels (Fig. 7). In some such cases its site and general form make its diagnosis easy thus indicating the nature of other masses of elastic tissue which may not be so characteristically disposed and, furthermore, it often helps in recognition of various parts of the tissues apart from staining. The elastic tissue fluoresces a yellow colour which is quite distinct from the green of the fluorescein-globulin stain. Some of the mucins, although they fluoresce well because they stain with the fluorescein-globulin, sometimes show a small degree of a bluish fluorescence. This applies to the mucins of both epithelial and connective tissue origin. Although not of connective tissue, it might be mentioned here that keratin also has a bright blue-white auto-fluorescence (Fig. 6). In all such cases the fluorescence is completely independent of the phenomena being discussed here, but must be clearly recognized in all applicable tissues so that it may be excluded from consideration of the fluorescence due to the presence of the stain.

The connective tissue cells which stain with the fluorescein-globulin have been principally histiocytes, plasma cells, enlarged endothelial cells and foreign body giant cells (Fig. 13, 14). In general, the small round cells of the lymphocyte type do not obviously fluoresce, but the larger cells—the macrophages—fluoresce brightly ; this is particularly apparent when these occur in groups. In addition to these mononuclear cells, polymorphonuclear leucocytes also stain (Fig. 1, 2).

Examination of blood films shows that in normal circumstances red blood corpuscles do not stain but cells of the white series of all kinds do (Louis, 1957*d*) and this applies also to the mononuclear cells of the blood including the small round cells (Fig. 1, 2). The lymphocytes require further study because of some apparent discrepancy between those in the blood stream and in the tissues. The apparent difference may not necessarily be a real one but may well be due to the relative difficulty in demonstrating fluorescence of the small amount of material in sections as compared with the relative ease of observing such changes in a film.

Occasionally some cells which might be expected to stain (or which, in other circumstances, are found to stain well) fail to do so. It is well known that fluorescence of tissues may be damped down or obscured by specific materials present. This may be due to some physico-chemical change induced in the material which previously fluoresced, or it may be a simple physical blanketing of the fluorescent material. A good example of this is some of the cells containing melanin pigment (Fig. 6). Most of the pigment-producing and pigment-containing cells in the skin fluoresce well, but occasionally some of these do not and it is found that this is associated with the presence of a relatively larger amount of pigment in the cells.

In these cases this lack of staining may be due to displacement of protoplasm by a product of tissue activity, or to an obscuring of the fluorescence phenomenon which is normally present. Investigations of cells from which the pigment has been removed chemically have not yet been made.

The problem, therefore, is one of the mechanism of staining. It is clearly not solely a matter of the type or origin of the cell. The one feature which appears to be common to the cells which stain is that they possess a considerable mass of cytoplasm. It is of no consequence whether the cells are epithelial or of connective tissue origin ; provided that there is a sufficient volume of cellular material this, in ordinary circumstances, will stain. If the amount of cytoplasm is small then the staining of any such material may not be recognized.

A point of interest here is the staining of endometrial tissue. The glands stain well in the normal fashion, that is, the cytoplasm stains though the nuclei do not but, in addition, the cells of the stroma stain similarly (Fig. 9, 10). This phenomenon may be considered from two points of view. In the first place the stroma cells are quantitatively similar to the cells of the glands, that is, they contain a considerable amount of cytoplasm, but there is also the question of the relation between the two kinds of cells. It has been frequently stated that the glands are " epithelial " whereas the stroma is connective tissue. From the purely morphological point of view this is true, but the implication that they are therefore different from each other is quite unjustified, and recent work has indicated that stromal cells can develop from glands, and glands grow and differentiate from stroma (Brines and Blain, 1943). It is unnecessary to discuss this point in detail here but it is clear that cells of different forms may show similar staining characteristics provided that they are similarly endowed with cytoplasm.

In all these cases it is the protoplasm of the cells which stain and the nuclei do not. This is very clearly seen in photomicrographs of, for example, the liver where the cells show a brightly fluorescing cytoplasm and an ovoid or round area of absence of fluorescence corresponding to the position of the nucleus (Fig. 5). In the case of red blood cells, however, the situation is reversed (Louis, 1958*a*). The haemoglobinized red cell does not stain but, in the lower vertebrates where all the red corpuscles are nucleated, the nuclei fluoresce brightly (Fig. 3). This kind of phenomenon would present considerable interpretative difficulties with the older idea of a serological reaction and antigen-antibody phenomenon but, in the light of the present concept, it would appear that in the nuclei of the red cells there must be some basic protein which will unite with the relatively acid fluorescein-globulin complex.

The failure of the red corpuscles to stain in normal circumstances presents, at first sight, a problem in that there is in the cells a considerable mass of material ; but at the same time there is relatively little cytoplasm comparable with that seen in other cells since haemoglobin is the predominant constituent and the isoelectric point of oxyhaemoglobin is below the pH at which staining is carried out. Since at this pH fluorescein-globulin complex and haemoglobin would have like charges, mutual repulsion rather than attraction of the two charged components could be expected.

The complexity of the problems is shown by the fluorescence of red blood corpuscles in certain circumstances. This occurs particularly in cases in which there appears to be some serological change of the kind seen in antigen-antibody phenomena (Louis, 1957*d* ; Hughes 1958*b*). Thus it is found in cases of acquired

haemolytic anaemia and in lupus erythematosus (Fig. 4). Observations have been made which indicate that, in this case, the phenomenon is one on the surface of the cell due to coating of the cell with protein which, in its turn, unites with the stain. This is quite a different phenomenon from the staining of the cytoplasm of cells of other tissues.

That the cells and tissues of the central nervous system failed to stain requires elucidation. This peculiarity was not unexpected in view of other features of staining of nervous tissue. The large amount of lipoid material in this tissue is doubtless significant as is readily recognized by all who have worked, in even a small way, with lipoproteins. Furthermore there is the observation that, in experiments carried out with insulin tagged with fluorescein, this has been shown to involve all cells with the exception of red blood corpuscles and cells of the central nervous system.

These cells therefore can be regarded as a special group which differ physically and chemically from those of other tissues in the body.

EXPLANATION OF PLATES

FIG. 1.—Fluorescence photomicrograph of a peripheral blood film (man) stained with fluorescein-protein complex and showing three fluorescing white cells. The red blood cells do not fluoresce. × 120.

FIG. 2.—Same area as shown in Fig. 1 subsequently stained by Leishmann's method for comparison.

FIG. 3.—Fluorescence photomicrograph of a peripheral blood film (fowl) stained with fluorescein-protein complex and showing one fluorescing white cell (below) and nuclei of red cells. × 160.

FIG. 4.—Fluorescence photomicrograph of a peripheral blood film taken from a patient with acquired haemolytic anaemia (positive Coombs test). All the red blood cells fluoresce. × 200.

FIG. 5.—Fluorescence photomicrograph of a section of rat liver stained with fluorescein-protein complex and showing a bright and uniform fluorescence of the cytoplasm of all the liver cells. The nuclei do not fluoresce. × 160.

FIG. 6.—Fluorescence photomicrograph of a section of a simple mole (man) stained with fluorescein-protein complex. The cells containing pigment absorb the fluorescence and thus do not become visible. The linear fluorescence beneath the epidermis is an auto-fluorescence (which was yellow as opposed to the green colour of the main tissue) due to elastic tissue. × 160.

FIG. 7.—Fluorescence photomicrograph of a section of mouse kidney stained with fluorescein-protein complex and showing fluorescence of the cytoplasm of the tubular cells. Note the linear (yellow) auto-fluorescence of the internal elastic lamina of the arteriole. × 240.

FIG. 8.—Same area as shown in Fig. 7 subsequently fixed in formol-saline and stained with haematoxylin and eosin for comparison. × 240.

FIG. 9.—Fluorescence photomicrograph of a section of endometrial scrapings stained with fluorescein-protein complex and showing fluorescence of both stromal and glandular cells. The vacuoles of the cells and the nuclear material take the place of cytoplasm and thus results in diminution of the brightness of staining. × 160.

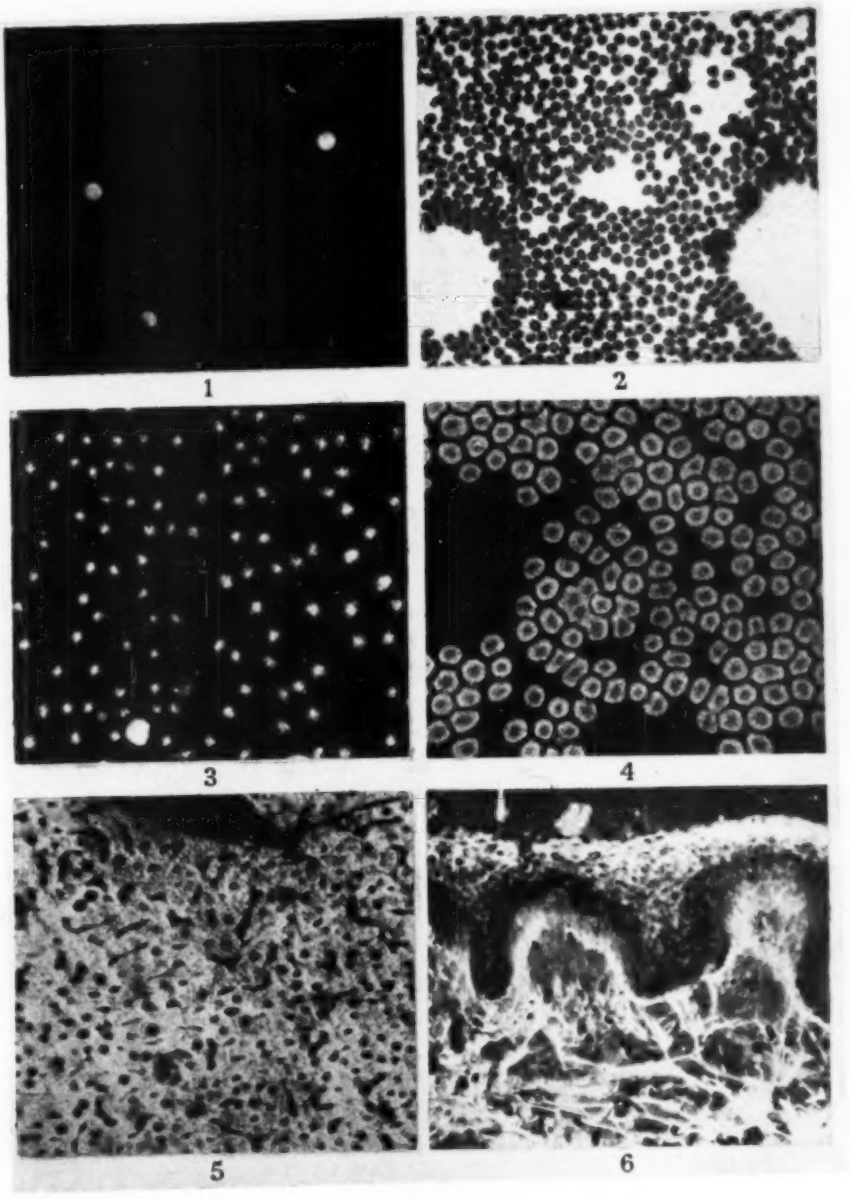
FIG. 10.—Same area as shown in Fig. 9 subsequently fixed in formol-saline and stained with haematoxylin and eosin for comparison. × 160.

FIG. 11.—Fluorescence photomicrograph of a section of a transplanted carcinoma of the breast (mouse) which is sharply demarcated from an area containing many plasma cells, macrophages and polymorphonuclear leucocytes. The inflammatory cells fluoresce brightly but the tumour tissue below fails to do so. × 320.

FIG. 12.—Same area as shown in Fig. 11 subsequently fixed in formol-saline and stained with haematoxylin and eosin to show the structure of the non-fluorescing area. A capillary vessel cut longitudinally separates the non-malignant from the malignant cells. × 320.

FIG. 13.—Fluorescence photomicrograph of a section of a spontaneous carcinoma of the breast from a lactating mouse. The overlying skin, glandular tissue and some striated muscle fibres fluoresce brightly but the tumour tissue below fails to do so. × 160.

FIG. 14.—Same area as shown in Fig. 13 subsequently fixed in formol-saline and stained with haematoxylin and eosin to show the structure of the non-fluorescing area. × 160.



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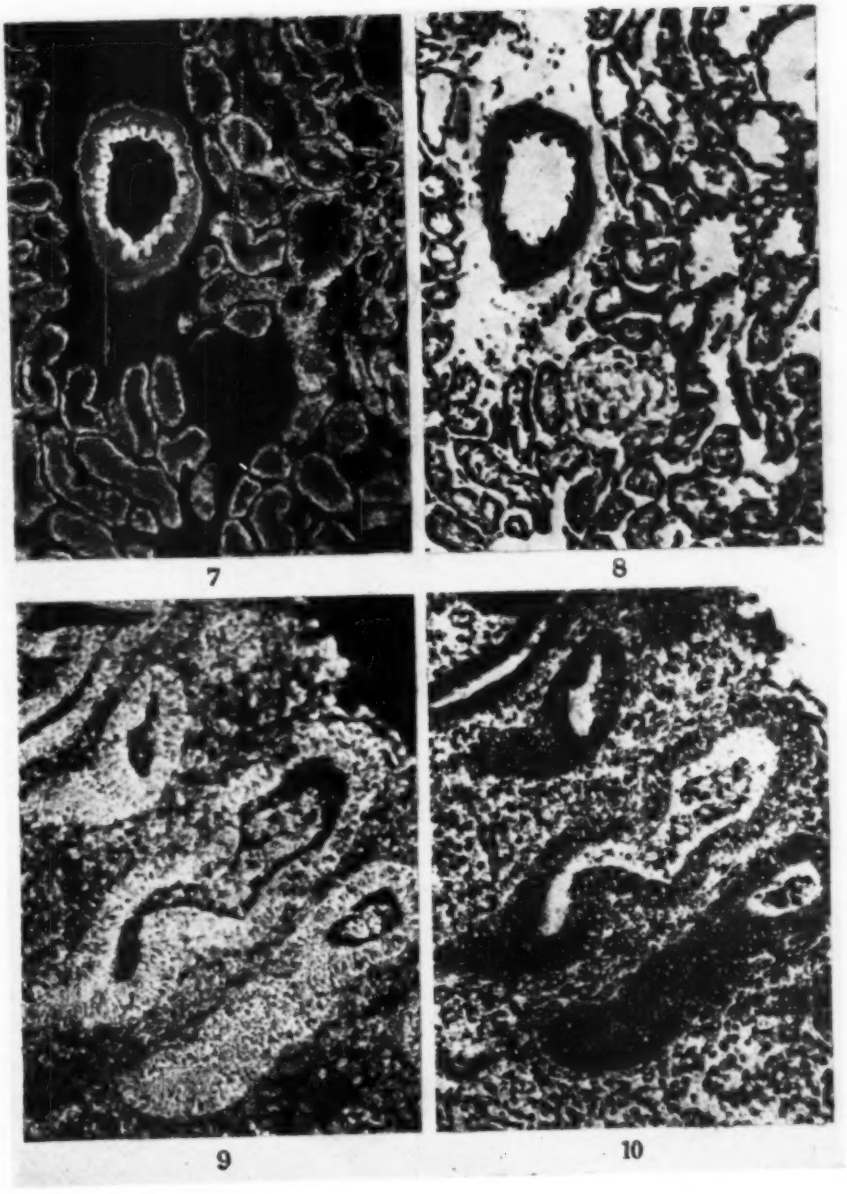

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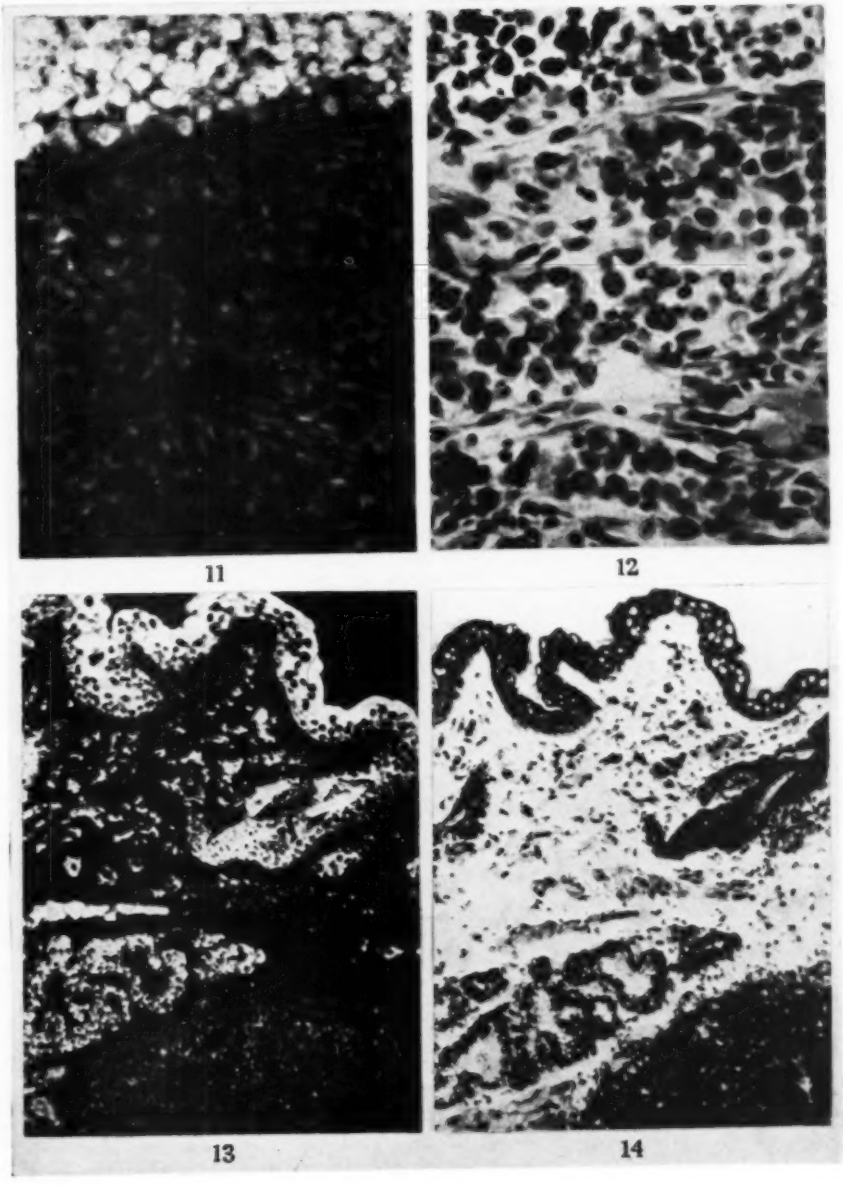

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Louis.

In resumé, therefore, a considerable proportion of the cells of the body stain with fluorescein-globulin in normal circumstances. These include all the cells of epithelium or parenchymal tissues, all connective tissue cells which have well-defined cytoplasm and this includes particularly the histiocytes and wandering cells, and the white blood corpuscles of the bone marrow and blood. The staining of all these cells occurs in the cytoplasm and their nuclei do not stain ; this staining depends on the presence in the cytoplasm of a basic protein which unites with the relatively acidic fluorescein-protein. The nuclei of nucleated red cells (particularly in the lower vertebrates) also stain and this phenomenon has not yet been elucidated.

There are certain circumstances in which normal cells do not stain. Ordinary connective tissue in which there is a very small amount of cytoplasm in relation to the connective tissue nuclei fails to show any significant staining and, indeed, from the point of view of ordinary routine examination such connective tissue can be said not to stain. There are circumstances in which the cytoplasm is replaced or obscured by intracellular components, a particularly good example being the occurrence of melanin in pigment-producing cells and these cells will not stain.

It can be said, therefore, that all the normal cells of the body which contain a significant amount of cytoplasm, with the exception of certain well-defined examples (the reason for their differences being susceptible to simple explanation) stain with these fluorescein-protein stains in a similar manner.

When malignancy occurs there are demonstrable changes in the cells, one of which is the loss of a basic protein in the cytoplasm and thus these tissues fail to stain (Fig. 11, 12, 13, 14). This constitutes a striking phenomenon, particularly when portion of a tumour and some of the normal tissue of the area are present in the same section.

It is obvious that, in view of discrepancies in the assumption that staining of normal tissues is universal, all cases of non-staining tissue, assumed to be malignant, should be compared with a normal tissue of a comparable type. Such comparison is readily made in epithelial tumours and in those of tumours containing cells with a considerable amount of protoplasm.

In the case of the sarcomata, however, observations are not so significant. There is no question but that the sarcomata do not stain, but then original cells often fail to stain, but this is because they do not possess enough cytoplasm.

If we consider some of the reticulosarcomata where the prototype is a cell which has a moderate amount of protoplasm and which, therefore, ordinarily stains, we have a relationship similar to that seen in the case of the epithelial growths. Thus, though the method cannot be used with any degree of certainty, in the case of the sarcomata in general, if the tumour cells are relatively large and do contain cytoplasm, the demonstration of failure to stain has considerable significance ; however, here the conclusions are drawn from a general consideration of the characteristics of cytoplasm of cells rather than by a direct comparison between malignant tumours and the corresponding normal tissue.

Thus, when changes occur in connective tissue so that the cells become larger, for example, when fixed cells change their character and become well-defined bodies (wandering cells of the histiocyte type), they develop a clearly defined staining capacity, whereas when the change is in the direction of malignancy such staining capacity does not appear. Doubtless in due course, with improved

and more refined methods of staining, it may be possible to demonstrate some staining character in the connective tissue cells of the normal tissues.

A problem which is related to the above question, but which is on a less general plane, is that of the significance and relation to malignant tissues of hyperplastic tissues and innocent tumours. This will be the subject of a subsequent paper.

SUMMARY

Fluorescein-serum protein may be used as a serologically non-specific stain which stains normal but not malignant tissues.

All normal tissues of vertebrates (and some invertebrates) stain well and fluoresce brightly provided there is a sufficient amount of protoplasm in the cells.

This phenomenon is due to the presence in the normal cell of a protein complex whose isoelectric point is sufficiently different from that of the serum proteins to allow protein-protein interaction.

Normal cells which fail to stain are :

(i) Resting connective tissue cells which have considerable intercellular substance but little cellular proteoplasm, such as a normal fibroblast ;

(ii) cells containing materials such as melanin pigment which obscures the protoplasm of the cell ;

(iii) red corpuscles where haemoglobin (the isoelectric point of which is too near that of the proteins used in the stain to allow combination) constitutes a major part of the cell material.

In all cases malignant tissue fails to stain. The significant feature is that the cells of such a tumour contain cells with, often, voluminous protoplasm but this does not show the phenomenon.

The conclusion that non-malignant cells may be distinguished from normal cells has been demonstrated without real exceptions (excluding the normal haemoglobinized red cell). Scrutiny of the few exceptions indicates that the differences are of a form and degree (as indicated by the lymphocyte) that will be resolved with improvements in technique.

(iv) Cells of the central nervous system which can be regarded as constituting a special group.

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OBSERVATIONS ON THE EFFECTS OF CONDENSATES FROM CIGARETTE SMOKE ON HUMAN FOETAL LUNG *IN VITRO*

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In previous experiments it was shown that 3–4-benzpyrene when added to the culture medium induced changes of a precancerous nature in organ cultures of human foetal lung. These consisted of hyperplasia of the bronchiolar epithelium with irregular increase in nuclear size, hyperchromatosis and abnormal cell divisions.

The present work is an attempt to examine by the same method the effect of condensates from cigarette smoke on human foetal lung in culture.

MATERIAL AND TECHNIQUE

The smoke condensates† were kindly supplied by the late Sir Ernest Kennaway. All but one batch were derived from Denicotea filters and prepared by Mrs. G. Lewis, Mrs. M. Urquhart and Dr. J. M. Campbell, then all members of Sir Ernest Kennaway's Department at St. Bartholomew's Hospital, London, whose help in this investigation I gratefully acknowledge, while one batch was derived from a smoking machine.

The neutral fractions of the condensate from which nicotine and phenols had been removed were used throughout the experiments. After evaporation of the solvent, either acetone or cyclohexane, they were freeze dried and homogenised with sterile horse serum. The freeze drying made the waxy condensates brittle and facilitated the homogenising process. The resulting suspension was kept at 4° C. and diluted to the desired concentration with human serum directly before use.

Four variations of the neutral fraction were used :

1. The whole of the neutral fraction (solvent acetone) (*a*) from Denicotea filters, (*b*) from a smoking machine.
2. Neutral fraction from Denicotea filters (solvent cyclohexane). This contains approximately 90 per cent of the hydrocarbons present in the neutral fraction.
3. Neutral fraction from Denicotea filters, from which the hydrocarbons had been removed by column chromatography.

The lungs were obtained from 3–5-months-old foetuses after surgical terminations of pregnancies. They were removed aseptically and cut into fragments of 1 × 2 × 4 mm. in size and explanted on to the surface of a clot consisting of chick

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† This term, first used by Sir Ernest Kennaway, describes the product of cigarette smoke more aptly than the more widely used term " tobacco tar ".

plasma, human serum and 50 per cent chick embryo extract in a proportion of 4 : 2 : 2. The clot was contained in a watchglass which was enclosed in a Petri dish carpeted with damp cotton wool to prevent evaporation (Fell and Robison, 1930). The total amount of medium per watchglass was 0·75 ml.

The condensate was added to the medium before clotting in a concentration of 300 μg./ml. of total medium. The explants were transferred to a fresh medium with fresh condensate every 3–4 days and were fixed in 3 per cent Zenker's fluid at 7, 10, 12, 13, 16 and 19 days' growth. For easier tabulation they were grouped into sets fixed after 7–10, 12–13, and 16–19 days.

After fixation the cultures were dehydrated, embedded in paraffin and serially sectioned at 6 μ. The sections were stained with haematoxylin-eosin, with Schiff's periodic acid method and with carmalum-orange G, anilin-blue (modified Azan).

RESULTS

Control Explants

The growth of the untreated lung explants has been described previously (Lasnitzki, 1956) and will only be shortly summarised here. Living explants become surrounded by an outgrowth. of translucent branching bronchioli while isolated fibroblasts and macrophages wander out from the explant towards the periphery of the clot. Histological examination of serial sections shows formation of new bronchioli at the periphery as well as in the centre of the explant. They are embedded in cellular connective tissue and lined with one row of cuboidal or cylindrical secretory epithelium (Fig. 1 and 2). Bronchial cartilage develops in many cultures : it is first discerned as a focus of condensed mesenchymal cells which after two to three weeks' cultivation differentiate into typical cartilage.

Effects of Smoke Condensates

All four subfractions promoted the growth of the bronchiolar epithelium (Table I) but the degree, extent, induction time and the histological type of the hyperplasia varied considerably with the different fractions used.

Two main types of changes could be distinguished : generalised increase in formation of bronchioli was seen after short periods of treatment. The newly formed bronchioli were still lined with one row of cells but these were frequently

TABLE I.—*The Number of Treated Cultures Showing Growth Promotion and Hyperplasia*

Time of treatment (days)	Whole neutral fraction: Denicotea cond.	Whole neutral fraction: Smoking machine cond.	Subfraction containing mainly hydrocarbons	Hydrocarbon-free subfraction
7–10	2/9*	7/13	13/18	—
12–13	4/16	2/8	6/10++	2/7
16–19	7/23+–	5/8+	5/14+	12/22++

* The right-hand figures give the total number of cultures fixed at each point of observation.
+– Slight hyperplasia.
+ Moderate hyperplasia.
++ Extensive hyperplasia.

increased in height, particularly in bronchioli occupying the periphery of the explant (Fig. 3); hyperplasia of the bronchiolar epithelium occurred usually after the longer times of application and was more often seen in the larger bronchioli. The increased cell proliferation progressed from the periphery towards the centre of the bronchus and often led to partial or complete occlusion of the lumen.

The epithelial hyperplasia was of three different histological types: (1) basal cell hyperplasia in which small crowded basal cells were lined by an intact inner layer of columnar epithelium which was secreting considerably more than that in the controls; (2) loss of the secretory epithelium and pleomorphism of cells with irregular enlargement of the nuclei; (3) squamous metaplasia, also with loss of the secretory epithelium which was replaced by flattened cells with their long axes parallel to the lumen. The epithelium resembled the epidermis, except that there was no keratin formation.

Effects of whole neutral fraction

(a) *Denicotea condensate.*—This substance was only mildly active; after 7–10 days' treatment 2 out of 9 explants showed increased formation of new bronchi, after 12–13 days 4 out of 16 cultures exhibited similar changes while after 16–19 days in 7 out of 16 cultures a small number of bronchioli with basal cell hyperplasia were observed.

(*b*) *Smoking machine condensate.*—This was more effective than the Denicotea condensate. After 7–10 days' exposure, 9 out of 13 cultures showed increased bronchus formation and in 2 of these hyperplastic bronchioli were found as well. After 16–19 days' treatment, 5 out of 8 cultures showed both changes: i.e. more newly formed bronchi as well as increased proliferation of the lining epithelium. The hyperplastic epithelium consisted mainly of irregularly enlarged crowded cells with hyperchromatic nuclei; the secretory cells were lost (Fig. 4) and in a few bronchi there was a transition to squamous metaplasia.

Neutral fraction containing mainly hydrocarbons.—After 7–10 days' treatment 12 out of 18 cultures showed increased formation of new bronchi. After 12–13 days both changes, a generalised increase in new bronchioli as well as extensive basal cell hyperplasia of individual bronchioli were present in 6 out of 10 cultures. The hyperplastic epithelium consisted of 6–12 rows of densely crowded basal cells with abundant mitosis and was lined with intact functioning, secretory epithelium (Fig. 5, 6). After 16–19 days' treatment 4 out of 14 explants showed hyperplasia of a moderate degree.

Neutral fraction without hydrocarbons.—In this set of experiments the cultures were fixed after 12 and 19 days's exposure. After the shorter growth period 2 out of 7 explants showed increased bronchus formation, but after 19 days marked hyperplasia of the larger bronchioli was present in 12 out of 22 treated cultures. In these the epithelium had multiplied to 6–10 rows of cells which became stratified and had undergone squamous metaplasia (Fig. 7, 8).

DISCUSSION

Since a statistical relationship between the increase of human lung cancer and smoking was first established (Doll and Hill, 1954, 1956; Hammond and Horn, 1954) many investigators have attempted to find experimental evidence for carcinogenic properties of cigarette smoke.

Cooper and Lindsey (1955) demonstrated the presence of small amounts of 3–4-benzpyrene and 1–12-benzperylene in the neutral fraction of cigarette smoke condensate. These findings were confirmed by Wright and Wynder (1955), Lettré, Jahn and Hausbeck (1956), and Bonnet and Neukomm (1957) who in addition isolated two other carcinogenic hydrocarbons, 1·2-benzanthracene and 3–4–9–10-dibenzpyrene from the neutral fraction.

Of the many workers who tried to induce animal tumours by application of cigarette smoke in various forms, Wynder, Graham and Croninger (1953, 1955) and Wynder, Kopf and Ziegler (1957) reported the production of skin carcinomas in various strains of mice painted for long periods with concentrated condensate, and Graham, Croninger and Wynder (1957) obtained cancer in rabbits' ears treated similarly. Blacklock (1957) produced one carcinoma and one sarcoma in the lungs of rats injected with a mixture of Denicotea condensate and killed tubercle bacilli. In all three experiments the induction period corresponded to over half the natural life span of the animals while the incidence of carcinomas varied from 3 to 44 per cent.

Gellhorn (1958) found that a combination of 3–4-benzpyrene and smoke condensate produced significantly more skin carcinomas in mice than either compound alone, a result which suggests either an additive effect due to the carcinogenic substances contained in smoke or a cocarcinogenic action.

Rockey *et al.* (1958) showed that tobacco tar applied to the bronchial mucosa of dogs induced hyperplasia and squamous metaplasia within a few weeks but there was no increased mitotic activity and no atypism. Leuchtenberger, Leuchtenberger and Doolin (1958) also observed hyperplasia and squamous metaplasia of the bronchial epithelium of mice exposed to cigarette smoke ; the hyperplastic epithelium was occasionally atypical and showed graded increases in nuclear volume, dry mass and DNA.

In the present experiments smoke condensate added to the medium of growing human foetal lung promotes the growth of the bronchiolar epithelium after an exposure of 10 to 19 days. At first a generalised increase in the formation of new bronchioli is observed ; this is followed by hyperplasia of the lining epithelium in individual bronchioli. The number of cultures affected, the extent of hyperplasia in them and its histological type varies with the different fractions used.

The early generalised growth-promotion is of the same histological type with all three fractions while the second stage—the hyperplasia of individual bronchioli —can be divided into three distinctive histological patterns : (1) basal cell hyperplasia with preservation of functioning secretory epithelium occurs after administration of the hydrocarbon fraction ; (2) loss of the secretory epithelium, irregular enlargement and hyperchromatosis of nuclei, is seen frequently after the whole fraction from smoking-machine condensate, while (3) squamous metaplasia also with loss of the secretory epithelium, is observed after treatment with the hydrocarbon-free fraction. Mitotic abnormalities are seen after all three fractions but are most frequent after the smoking machine condensate.

The epithelial hyperplasia resembles that observed after treatment with 20-methylcholanthrene in organ cultures of the mouse prostate and 3-4-benzpyrene in human foetal lung (Lasnitzki, 1951, 1956) except that the latter substance only rarely caused squamous metaplasia.

The identification of 3–4-benzpyrene in the neutral fraction of smoke condensate has focused attention on this compound as the causative agent in the carcinogenesis

of lung tumours. Previous work has shown that the minimum dose of 3–4-benzpyrene necessary to induce epithelial hyperplasia of the bronchial epithelium under identical experimental conditions was 1 μg./ml. of medium applied for 4 weeks. In the present experiment the concentration used per ml. of medium was the yield of 0·2 cigarettes containing according to Cooper's and Lindsey's data (1955) 4^{-3} μg. of 3–4-benzpyrene, an amount far too low to account for the changes seen. It must be assumed therefore that they are due, at least as far as the hydrocarbon fraction is concerned, to the sum of all carcinogenic hydrocarbons present in the neutral fraction.

The fact that the non-hydrocarbons also stimulate the growth of the bronchial epithelium suggests that in addition to the hydrocarbons other compounds must play a rôle, either as causative or promoting agents.

Whether the hyperplasia induced experimentally *in vitro* would ultimately lead to true malignancy under the more complex conditions *in vivo* is not certain, but the results can nevertheless be considered a useful guide and suggest that the neutral fraction from smoke condensate may be carcinogenic to the human lung.

SUMMARY

The effect of condensates from cigarette smoke was studied on organ cultures of human foetal lung.

Four different fractions were used : the whole neutral fraction from Denicotea condensate, the whole neutral fraction from a smoking machine, and two sub-fractions of the neutral Denicotea fraction, one containing mainly hydrocarbons, the other without hydrocarbons.

Control explants showed outgrowth of branching bronchioli lined with one row of cuboidal or cylindrical secretory epithelium.

All four condensates increased the formation of new bronchioli and induced hyperplasia of the lining epithelium in individual bronchioli leading to partial or complete occlusion of the lumen.

The changes were least marked after the whole neutral fraction from Denicotea condensate and most extensive after the hydrocarbon fraction.

The hyperplastic epithelium showed three different histological types depending on the fractions used.

Basal cell hyperplasia was seen after the whole neutral fraction from Denicotea condensate and after the hydrocarbon fraction, pleomorphism of cells with loss of the secretory epithelium was present after the neutral smoking machine fraction, while squamous metaplasia was observed after the hydrocarbon free fraction.

The changes resemble those obtained after treatment with 20-methylcholanthrene and 3–4-benzpyrene in organ cultures of the mouse prostate and human foetal lung, except that the latter substance rarely caused squamous metaplasia.

The very low amount of 3–4-benzpyrene present in the neutral fraction together with the finding that the hydrocarbon free fraction also produces hyperplasia indicates that the effects cannot be due to 3–4-benzpyrene alone.

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EXPLANATION OF PLATES

FIG. 1.—Section through a control culture after two weeks' growth *in vitro*, showing bronchioli embedded in cellular connective tissue. Haematoxylin-eosin. × 97.

FIG. 2.—Section through another control culture after the same growth period *in vitro*. The bronchioli are lined with one row of cuboidal or cylindrical secretory epithelium. Modified Azan. × 280.

FIG. 3.—Section through a culture grown for 10 days with the hydrocarbon fraction showing an increased number of bronchioli and enlargement of epithelial cells in some bronchioli. Periodic acid Schiff. × 97.

FIG. 4.—Section through a culture grown for 16 days with smoking machine condensate, showing hyperplasia of the bronchial epithelium. Note loss of secretory epithelium and irregularity of nuclear size. Haematoxylin-eosin. × 420.

FIG. 5.—Section through a culture grown for 13 days with the hydrocarbon fraction, showing marked hyperplasia of the basal cell type. Periodic acid Schiff. × 80.

FIG. 6.—Part of the same culture at higher magnification. Note abundance of mitosis and secretion. Periodic acid Schiff. × 230.

FIG. 7.—Section through a culture grown for 19 days with the hydrocarbon-free fraction. Haematoxylin-eosin. × 160.

FIG. 8.—Section through another culture treated for 19 days with the hydrocarbon-free fraction. Note transition to squamous metaplasia and loss of secretory epithelium. Haematoxylin-eosin. × 240.

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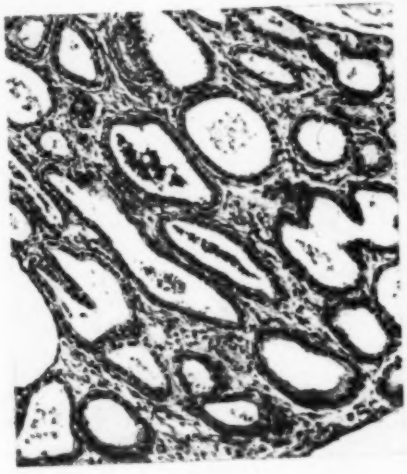

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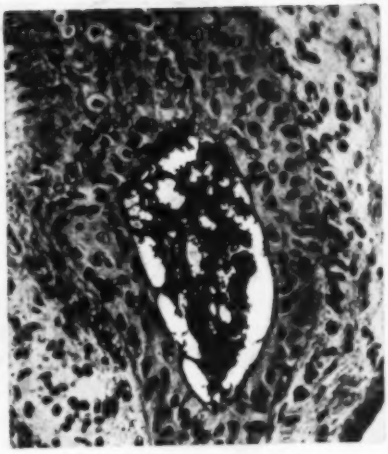

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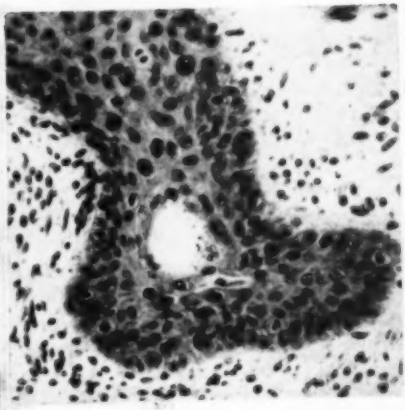

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THE HISTOGENESIS OF EXPERIMENTAL MOLLUSCUM SEBACEUM

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WHILE painting the skin of rabbits with the carcinogen 9 : 10-dimethyl-1 : 2-benzanthracene for the purposes of another experiment, it was noted incidentally that many of the regressing tumours so produced bore a close gross and histological resemblance to molluscum sebaceum in man (Heslop, 1956, 1958). This observation has also been made recently by Whiteley (1957) and Ghadially (1958). The fact that regressing tumours of skin may be produced by the application of carcinogens has been known for some time (Woglom 1926 ; Seelig and Cooper, 1933 : Rous and Kidd, 1939) although the existence of a very close counterpart in man was not appreciated until recently. Molluscum sebaceum was first described in the English language in 1936 (MacCormac and Scarff) but did not attract widespread attention till the last decade, during which time numerous clinical and histological accounts of the condition have been recorded (Rook and Whimster, 1950 ; Beare, 1953 ; Fouracres and Whittick, 1953 ; Calnan and Haber, 1955 ; Liban and Lennox, 1955 ; Whittle and Davis, 1957). The existence of the clinical condition was thus virtually unknown at the time when regressing papillomas in animals were first described.

Both in molluscum sebaceum in man and the similar experimental lesion in rabbits the histological appearances suggest an origin from hair follicles (Calnan and Haber, 1955 ; Whiteley, 1957 ; Ghadially, 1958). However, the exact sequence of changes leading up to the development of the established lesion has not been demonstrated. This is no doubt due to the fact that biopsies have been taken mainly from tumours which are identifiable on naked eye examination, whereas the earliest lesions are unlikely to be visible in the gross.

It was noted in the present experiment that the appearances in the area painted with carcinogen were constantly changing, with growth of some lesions and regression of others. This applied not only to the obvious tumours but also to some of the smaller nodules. These latter were barely classifiable as tumours in the gross, and presented rather as a coarse roughening or granularity in the skin. As these smaller nodules appeared and regressed in the same way as the larger tumours during the course of carcinogenic stimulation, it seemed probable that they were variants of the same process. On the assumption that the roughness and nodularity represented early or abortive forms of the larger tumours, it was decided to examine sections of the whole painted area with a view to establishing the histogenesis of experimental molluscum sebaceum. Accordingly apart from a few early biopsies confirming the nature of the gross tumours, histological

examination was concerned with the changes affecting the whole painted area rather than individual tumours.

METHODS

A circumscribed area of rabbit's ear was painted twice weekly for 20 weeks with an 0·5 per cent solution of 9 : 10-dimethyl-1 : 2-benzanthracene in benzene. A total of 45 Cross Dutch and Copenhagen White rabbits was used. In 14 animals a hole 0·5 cm. in diameter was punched in the treated area, at periods varying from 3 to 6 weeks after painting had commenced. The hair was shaved prior to the commencement of painting, but was not subsequently interfered with. Biopsies were taken from a few of the tumours during the course of painting. All the animals were killed at 20 weeks. The ears were fixed in formol saline and large sections cut across the whole painted area. Histological material was embedded in paraffin and sections were stained with haematoxylin and eosin.

RESULTS

Gross appearances

Generalised roughness was visible in the painted area within 17 days in all cases. This sometimes assumed the form of a diffuse scaliness of the skin. More often however the skin showed a fine or coarse nodularity, the spherical nodules appearing to lie beneath the skin surface. Both of these changes were invariably accompanied by apparent diminution in the density of the hair with matting of the remaining hairs, although total epilation was never observed. Sometimes a small pore was visible in the centre of a nodule, and matted hairs could occasionally be seen emerging from the pore. Nodules of this type, often in large numbers, were visible in all animals at this stage of painting. The exact stage at which either the nodules or the scaly patches became distinguishable as "tumours" seemed rather arbitrary, a fact which was later born out by the histological findings. For convenience lesions over 0·2 cm. in diameter were classified as tumours although it was appreciated that they merged imperceptibly with the smaller lesions already described. The tumours showed three main forms. namely :

(1) A raised nodule with smooth rolled edges and a central plug of keratin. Sometimes this showed a prominent keratinous horn. The latter was friable and tended to break off flush with the skin edges or even a little below them. This gave the impression of central ulceration. These tumours measured up to 1·5 cm. in diameter and closely resembled molluscum sebaceum in man.

(2) Single keratinous horns without obvious raised skin edges at the margins. These sometimes presented as clusters of several adjacent horns in which case the whole lesion showed a warty appearance. These were usually a little smaller than first type of lesion.

(3) Flat plaque-like lesions. These were not infrequently seen in the centre of a disced area which had become organised and covered with fresh epithelium. These were generally smaller than either of the previous two lesions.

Towards the end of the period of painting some of the animals showed numerous tumours which were often confluent. The larger lesions often showed a combination of the macroscopic features of all three above types of tumour, the individual components not being sharply separable. In addition to the above tumours two

rabbits showed clinically malignant tumours, with penetration of the cartilage and involvement of the other side of the ear.

Forty rabbits showed tumour formation during the course of painting, and regression of one or more tumours or smaller nodules was noted in all animals by the end of the 20-week period. Regression was observed in all types of tumour with the exception of the two malignant tumours. It was most frequently seen in the smaller tumours (less that 0·5 cm. in diameter) but this may have been due to the fact that these were more numerous than the larger lesions. Some of the small nodules (0·2 cm. in diameter and less) were observed to regress. To what extent regression occurred at an early stage was difficult to assess with certainty on naked eye examination alone.

Histological appearances

The earliest changes consisted of slight hyperplasia of the surface epithelium which was paralleled by similar hyperplasia of the epithelium of the upper part of the hair follicles (Fig. 1). This was often accompanied by dilatation of the upper part of the hair duct, the enclosed hair being surrounded by circumferential layers of keratin. Adjacent enlarged hair follicles soon coalesced (Fig. 2) the resultant cyst containing several hairs. One particularly large cyst of this type was seen to contain over 40 hairs (Fig. 3). The sequence of events leading to the development of the large cysts could often be traced from the keratin pattern within them. In each case keratin was initially laid down circumferentially round individual hairs. As fusion of the enlarged follicles occurred, groups of hairs came to be surrounded by larger rings of keratin produced by the epithelium of the cyst wall. Further coalescence resulted in the enclosure within circular deposits of keratin, of progressively larger aggregations of hairs. Fresh keratin was always laid down parallel to the cyst lining, and reflected the size of the cyst at the time of production of the particular sheet of keratin. Thus in the large cysts, the manner of development was often indicated by the character of the contained keratin. The cysts opened on to the surface through a pore of varying diameter. This fact no doubt explained both the apparent reduction in the number of hairs and the matting seen in the gross specimen, and also some of the small subepithelial nodules seen on naked eye examination. Where the cysts opened on to the surface through a wide mouth, large deposits of keratin were extruded (Fig. 4) and were seen in the gross as conical horns projecting from the surface. All the animals showed surface epithelial hyperplasia and changes in the hair follicles of at least the degree shown in Fig. 1.

In 40 of the rabbits the lesions were considerably more advanced. Changes leading to the development of macroscopic tumours tended to occur focally, and consisted of a further proliferation of either the surface epithelium, the epithelium lining the hair follicles or both in combination. Hyperplasia of the hair follicle epithelium sometimes occurred in the absence of much duct dilatation. Usually however, dilatation was marked and there was obvious cyst formation. Sebaceous glands were only occasionally visualised in relation to the cysts, and it seemed that the gland had either disappeared or had undergone squamous metaplasia and become incorporated into the cyst. Where follicular dilatation was less marked, the sebaceous glands were often more obvious and occasionally showed hyperplasia.

In the early stages epithelial hyperplasia in the hair follicles only involved the upper part. At a later stage it extended to involve the deepest parts of the follicle, the specialised features of which were no longer identifiable. In the latter case hairs were sometimes present in the upper part of a central core of keratin, which frequently showed the characteristic pattern already described. The keratin nearer the epithelial lining of such a cavity was laid down parallel to the surface and did not necessarily reflect the origin of the lesion in a number of hair follicles (Fig. 4, 5). This type of lesion presented the histological characteristics of molluscum sebaceum. Whether or not it showed smooth rolled edges in the gross appeared to depend on the degree of extension laterally under the normal skin. This was probably related to the size of both the antecedent pilosebaceous cyst and its surface opening. Those tumours which in the gross showed a large keratinous horn without raised edges in the surrounding epithelium showed essentially similar histological features, differing from the classical molluscum sebaceum only in showing a larger surface opening and relatively less lateral extension under the marginal epithelium (Fig. 4). The apparent ulceration seen in the gross was usually not accompanied by any breach in epithelial continuity. It was merely a reflection of loss of superficial keratin from these lesions.

Pure papillomas were much less frequent than lesions derived from the hair follicles. Tumours were classified as papillomas when they consisted of localised proliferation of the surface epithelium, with variable development of papillary folding. Hair follicle involvement was absent or inconspicuous (Fig. 6, 7). These tumours were always raised above the surface to some extent, and appeared either warty or plaque-like in the gross. Some of the warty lesions were seen on histological examination to present apparent papillary folding of the surface epithelium, which was in fact due to the presence of several adjacent pilosebaceous cysts (Fig. 10) and not to pure surface hyperplasia. The keratin pattern was useful in identifying this fact. Intermediate lesions showing both surface hyperplasia and involvement of the hair follicles were much more numerous than pure papillomas (Fig. 8, 9).

In all types of tumour very pronounced epithelial hyperplasia was seen in some of the larger lesions. The thickened epithelium was often thrown into papillary folds. At a later stage infiltrative growth in the vicinity of the tumour was evident. The cytological features of this type of growth were not distinguishable with certainty from those of the two proven carcinomas in the series. The fact that pseudoepitheliomatous hyperplasia occurred in relation to tumours which were known to be regressing was apt to influence the interpretation of similar appearances in connection with other tumours. While most of the tumours were clearly benign from the histological point of view, the exclusion of malignancy did not appear possible in some others. This difficulty was noted particularly in those lesions where the infiltrative growth was not sufficiently extensive to have brought about penetration of the cartilage or ulceration of the skin (undoubted indications of malignancy), especially when the tumour had not already commenced to regress at the time of examination. This histological interpretation explains low incidence of malignancy in these experiments as compared with the classical work of Berenblum (1945, 1949) using this carcinogen in rabbits.

This series was mainly concerned in ascertaining the development of lesions, and the exact sequence of events leading to regression was not studied in detail. Judging by the occurrence of naked eye diminution of some of the smaller nodules,

it seemed that lesions could regress at an early stage. This fact was likewise suggested histologically by the presence of inflammation and epithelial degeneration in relation to some of the smaller pilosebaceous cysts, which were too small to be classified as tumours. Similar degeneration and inflammation were always associated with larger tumours which were known to be regressing at the time of examination.

DISCUSSION

In the early stages of carcinogenic stimulation hyperplasia of the surface epithelium is closely paralleled by hyperplasia in the upper part of the pilosebaceous follicle. It therefore seems not unreasonable to suggest that the later development of the papilloma from surface epithelium is analogous to the growth of the molluscum sebaceum from the pilosebaceous follicle. Additional support is given to this suggestion by the occurrence of lesions obviously derived from both components. When the analogy between the two lesions is appreciated it becomes clear that to a large extent the "invasive growth" characteristic of molluscum sebaceum is merely a reflection of cellular proliferation in an unusual site. Pseudo-epitheliomatous hyperplasia may occur in both lesions, but it is likely to appear more sinister when situated deeply in the dermis in association with a molluscum sebaceum, than in relation to a more superficial papilloma.

The invaginated contours of the classical molluscum sebaceum appear to be produced by the coalescence of several pilosebaceous cysts, the orifice of which is frequently small by comparison with the diameter. In a few lesions in the present series cyst formation was less prominent in the hair follicles than hyperplasia, particularly in some of the compound lesions. The latter partook of the nature of both papilloma and molluscum sebaceum in showing proliferation of both surface and hair follicle epithelium (Fig. 8, 9). The contours of a lesion of this kind were dependent entirely on the relative preponderance of the two components and the tendency to cyst formation in the pilosebaceous follicles. At one end of the scale was a tumour showing mainly surface hyperplasia with relatively inconspicuous follicular hyperplasia and little tendency to cyst formation (Fig. 8). This type of lesion not infrequently showed hyperplastic sebaceous glands. At the other end of the scale was the classical molluscum sebaceum, the margins of which showed some superficial epithelial hyperplasia. In view of the fact that the lesions all appeared to arise in similar circumstances there seemed nothing to be gained from minute histological subdivision. Therefore tumours were classified as molluscum sebaceum or papilloma according to whether hyperplasia involved predominantly the hair follicles or the surface epithelium. It seems possible that these compound lesions in the rabbit may be similar in nature to some of the atypical papillomas seen in man. The latter often show the structure of a squamous papilloma but with the additional feature of epithelial hyperplasia in the hair follicles, sometimes accompanied by a minor degree of dilatation (Fig. 11).

In addition to the histological evidence suggesting that molluscum sebaceum develops from the hair follicles, circumstantial evidence was provided by the fact that it was never seen to occur in the centre of a disced area. The disc was initially filled with blood clot which became organised, the surface ultimately being covered by a simple epithelium devoid of skin appendages. Papillomas not infrequently occurred in this central area, but molluscum sebaceum was seen only

at the margin where hair follicles remained. In this connection Ghadially (1958) mentions the fact that human molluscum sebaceum does not arise on the palm of the hand where hairs are absent. Experimentally the absence or relative diminution of hair follicles beneath the larger mollusca sebacea offered further confirmatory evidence of the participation of the hair follicles (Fig. 4, 5). An attempt is being made at present to produce molluscum sebaceum in the skin of rabbits following epilation by X-rays.

In view of the apparent participation of the pilosebaceous follicle in the development of experimental molluscum sebaceum, the retention of the latter term seems preferable to the other suggested alternatives. From the descriptive point of view the term kerato-acanthoma is non-specific and could equally well be applied to superficial papillomas.

The direct application of experimental findings in animals to clinical lesions in man is always hazardous. However, there is a considerable body of clinical and histological evidence to suggest that the lesion in man may represent a comparable process to that seen in the experimental animal. The human molluscum sebaceum occurs predominantly in those areas where skin cancers are most frequent. It also shows a predilection for a similar age group. It has been reported in tar, oil and arsenic workers and in people exposed to actinic radiation (Binkley and Johnson, 1955). The gross and histological appearances in man and animals closely parallel each other. An origin in the hair follicles has been postulated in man on histological evidence (Calnan and Haber, 1955). In the original English paper on molluscum sebaceum it was suggested that the lesion arose in a sebaceous cyst, a hypothesis which accords well with the findings in the present series. One wonders to what extent some of the simple epidermal cysts in man might be analogous to the experimental pilosebaceous cysts in representing a response to carcinogenic stimulation. The manner in which the keratin pattern indicated the contribution of individual hair follicles to the whole experimental lesion has already been described. This characteristic pattern was frequently, although not invariably present in the animal lesions. Fig. 12 is a human molluscum sebaceum showing a similar keratin pattern. Calnan and Haber's Fig. 6 likewise shows these features and Ghadially's Fig. 6 is also suggestive in this connection. The keratin pattern has not previously attracted attention in man, but in the light of experimental results it offers strong circumstantial evidence of the hair follicle origin of the human lesion. In some of the experimental lesions where large keratinous horns developed, the characteristic circular pattern persisted in the upper part of the horn, while the keratin nearest the epithelium was laid down parallel to it. It would thus seem that the earlier lesions are more likely to show the characteristic keratin pattern while the keratin is still contained within the cyst and has not yet been pushed too far distally.

The extent of epithelial hyperplasia in each molluscum sebaceum was varied. The impression was gained that papillary hyperplasia was most pronounced in those lesions showing a wide central pore. It seemed that in larger lesions opening through a small pore the pressure of retained keratin possibly limited papillary epithelial growth. The reverse picture was seen in those lesions showing a row of adjacent wide-marked pilosebaceous cysts (Ghadially's Type I kerato-acanthoma), the intervening walls between the cysts giving a markedly papillary appearance to the whole lesion (Fig. 10). In this type of tumour, the keratin pattern was found useful in identifying the nature of the lesion.

The hair growth cycle was at no stage taken into account in this experiment. However, two points in Whiteley's (1957) results appeared worthy of comment in relation to results obtained here. In the first place some of the lesions classified by him as papillomas would appear actually to consist of several adjacent wide mouthed pilosebaceous cysts. Ghadially (1958) has classified this lesion as a Type I kerato-acanthoma. Thus the stated differences in incidence of molluscum sebaceum and papilloma according to the stage in the hair cycle becomes less clearly defined. In the second place one wonders to what extent plucking of hairs may have acted as a Deelman phenomenon in the hair follicles. While the question of the relation of trauma to tumour growth is always a vexed one, the impression was gained in this series that the punching of discs in the ears did accelerate tumour growth. Accordingly it seems possible that repeated minor trauma involved in plucking hair could have acted in the same way.

SUMMARY

Lesions resembling human molluscum sebaceum were produced in the skin of rabbits following painting with the carcinogen 9 : 10-dimethyl-1 : 2-benzanthracene. The genesis of these lesions from hair follicle epithelium is illustrated.

In addition squamous papillomas and compound tumours showing features of both papilloma and molluscum sebaceum were seen. It is suggested that the molluscum sebaceum is the hair follicle analogue of the superficial papilloma. Evidence is presented which suggests that molluscum sebaceum probably arises in man under similar circumstances to those obtaining experimentally. Lesions in man resembling the experimental compound tumours are also shown to occur.

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EXPLANATION OF PLATES

FIG. 1.—Hyperplasia of surface epithelium and epithelium lining the upper part of some hair follicles. × 15.

FIG. 2.—Further hyperplasia of hair follicle and surface epithelium with coalescence of adjacent follicles. × 10.

FIG. 3.—Large cyst produced by coalescence of numerous hair follicles. × 30.

FIG. 4.—Molluscum sebaceum with relatively wide surface opening and large keratinous horn. × 10.

FIG. 5.—Molluscum sebaceum with relatively narrow surface opening. × 6.

FIG. 6.—Papilloma presenting a plaque-like gross appearance. This area was disced and had healed with partial regeneration of cartilage. × 13.

FIG. 7.—Papilloma on healed disc, showing classical papillary pattern and wart-like gross appearance. × 11.

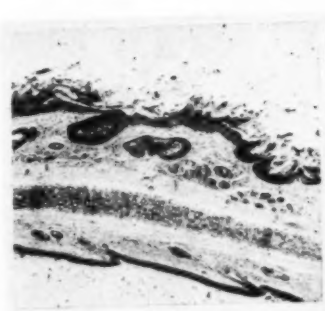
FIG. 8.—Compound tumour involving predominantly the surface epithelium. × 22.

FIG. 9.—Compound lesion with slightly more prominent hair follicle component. × 22.

FIG. 10.—Papillary appearance produced by several adjacent wide mouthed pilosebaceous cysts. × 27.

FIG. 11.—Warty lesion from man, present for some weeks. Hyperplasia of both surface and hair follicle epithelium, with circular keratin pattern on left. × 27.

FIG. 12.—Molluscum sebaceum in man showing circular arrangement of keratin reminiscent of experimental lesions. × 11.



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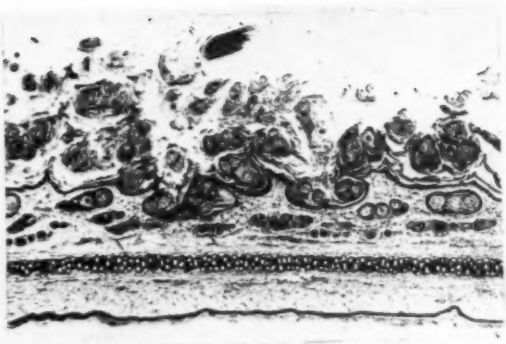

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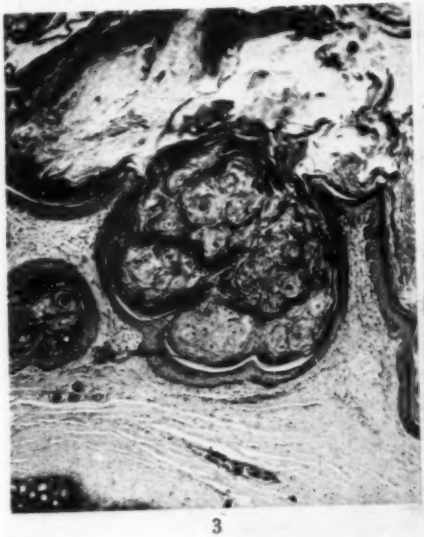

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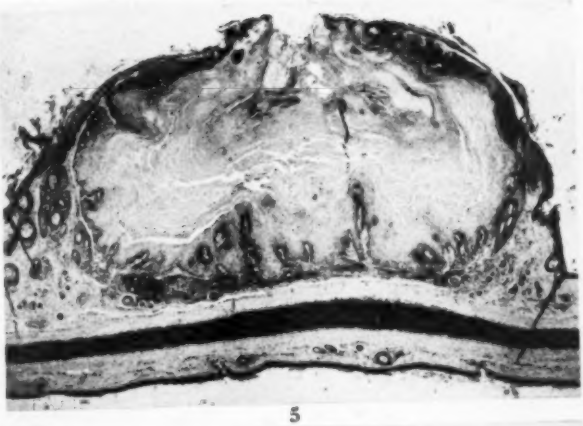

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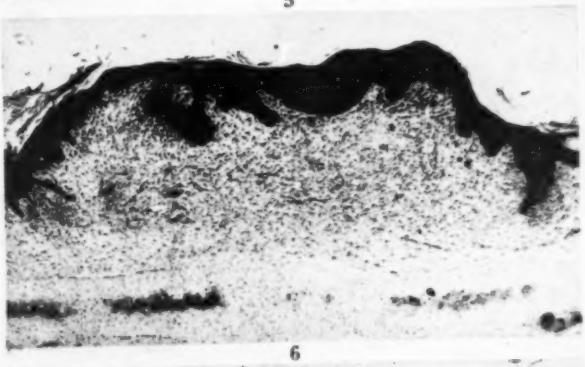

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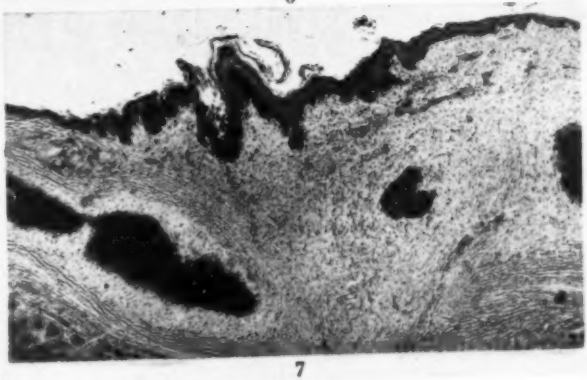

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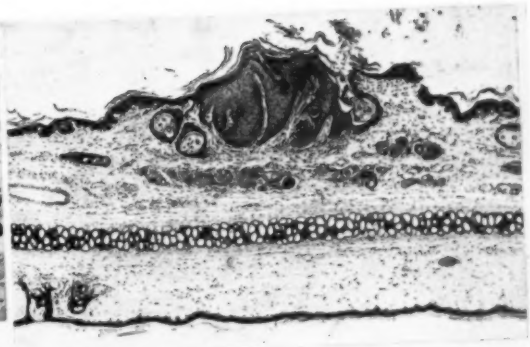

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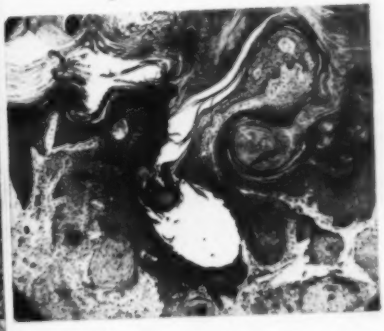

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RADIOSENSITIZATION OF THE ISOGENIC MAMMARY TUMOUR IN C3H MICE RECEIVING TISSUE GRAFTS OF VARIOUS ORIGINS

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AN appreciable increase in the radiosensitivity of the C3H mammary carcinoma was observed when the tumour-bearing mice were inoculated subcutaneously, about 2 weeks prior to treatment, with fragments of tumour or of isogenic neonatal skin previously irradiated with doses of 5000–7000 r (Cohen and Cohen, 1957). Since these isologous tissues are not normally antigenic, it was concluded that the attenuating procedure had changed some specific "identity markers" in the tumour and skin inocula, equivocating their isogenic pattern, thus evoking a type of homograft reaction in the host. This disturbance of the balance between the growing tumour and its host resulted in an enhanced response of the tumour to a comparatively small dose of radiation.

If radiation-attenuated isologous tissues act in this way, that is, by virtue of new antigenic constituents, resembling, but not identical with the tumour antigens, then unirradiated homologous tissues from other mouse strains or closely related species should also have a radiosensitizing action. The following experiments were designed to test this hypothesis.

MATERIALS AND METHODS

Experimental design

Young adult male mice of the C3H/Cg strain were used as tumour recipients in all experiments. Each of the 164 animals was inoculated subcutaneously in the right axillary region with a fragment of tumour introduced by means of a trocar and cannula. When the isografts became palpable about 2–3 weeks later, the second inoculum was minced finely with small scissors, either irradiated or not, as indicated, and implanted subcutaneously with trocar and cannula into the left flank. Eleven experimental categories, as listed in Table I, were investigated.

In the 3 groups receiving grafts of hybrid skin, neonatal mice with the following genotypes were used : (*a*) The F_1 offspring of a C3H male and a non-inbred stock albino female ; (b) Backcross mice, offspring of an F_1 hybrid female (from "*a*") and a C3H male ; and (*c*) Backcross mice from crossing an F_1 male (from "*a*") with a stock albino female.

In the 4 "homograft" groups, the skin was obtained from neonatal outbred albino mice, and spleen and kidney from young adults of the same stock. In one group of this series the skin was inoculated without further preparation in the manner described. In a second group, the skin was minced, placed in a plastic welled slide and irradiated with 7000 r prior to inoculation. A third batch of skin was minced and emulsified in an equal volume of Freund's adjuvant with *M.*

butyricum suspension (" Bacto-Adjuvant ", Difco) before inoculation. Spleen and kidney homografts were inoculated into smaller groups of 4 and 5 tumour-bearing mice respectively.

In the 2 " heterospecific " groups, skin was removed from non-inbred newborn stock albino rats, minced, irradiated in one instance, and inoculated as described.

Of the 2 " heterogeneric " groups, in one case skin was removed by surgical excision from an anaesthetized adult baboon, then minced and inoculated ; and in the other, 0·1 ml. of an emulsion of equal parts of normal saline and Freund's adjuvant with *M. butyricum*, was injected by hypodermic syringe.

Irradiation technique

The standard procedures for irradiation of the tumours *in situ*, and of minced tissues *in vitro* prior to inoculation, have been described in detail in previous papers (Cohen and Cohen, 1953, 1957).

All irradiated inocula received 7000 r after being minced and sealed in a plastic welled slide.

The tumours were treated *in situ* with a standard test dose of 4200 r when they had attained a diameter of about 1 cm., that is 4–7 weeks after implantation, and 2–5 weeks after the second inoculation. The expectation of cure at this dose, without preparatory sensitization, is about 0·1 per cent, so that two or more cures in any single experimental group may be taken as indicative of a significant induced radiosensitization.

RESULTS

The results of all the experiments are shown in Table I. The first three items included for comparison, are from previous data. It can be seen that living homologous skin from closely related donors, F_1 hybrids and backcross mice, as well as other homologous tissues, and even heterologous skin from a related genus, did in fact succeed in sensitizing the tumour.

Although the difference in the cure-rate between the hybrid graft groups (16 per cent) and the homograft group (32 per cent) is not statistically significant, a trend becomes apparent, on grouping the data, which indicates that the cure-rate of the tumour is influenced by the particular degree of antigenic dissimilarity between the host's tissues and the inoculum. Thus, unirradiated isologous skin which is by nature non-antigenic to the host, is not effective as a radiosensitizer (0/22 cures), whereas hybrid grafts containing both isologous and homologous protein components are somewhat more effective (16 per cent cures). One can assume that the quantities of the genetic components in the hybrids varied, that is, that the inocula from the F_1 group were half homologous, and those from the (C3H $\times$ F_1) backcross and the (F_1 $\times$ albino stock) backcross mice were one-quarter and three-quarters homologous respectively, yet no significant difference in cure-rates among the recipient mice could be demonstrated. This seems to indicate that the mere presence of the homologous component, irrespective of its proportional concentration in each genetic combination, was the deciding factor.

The overall cure rate in the homograft group was 32 per cent, with no significant differences between the irradiated and the unirradiated skin homografts (in contrast with the difference between irradiated and unirradiated isografts), and, as has been previously noted (Cohen and Cohen, 1957), the addition of Freund's

TABLE I.—*Radiosensitization of the C3H Mammary Tumour Induced by Tissue Grafts of Various Origins*

Sensitizing inoculum	Dose *in situ* 4200 r: Number of mice	Number cured	Grouped totals: Type	Grouped totals: Cures %
Total uninoculated controls*	108	0	—	—
Irradiated C3H tissues and tumours*	208	62	—	—
Unirradiated isologous (C3H) skin*	22	0	Isografts	0
(C3H × stock albino) F_1 hybrid skin	21	2	Hybrid grafts	16
(C3H × F_1) backcross skin	11	3		
(F_1 × stock albino) backcross skin	12	2		
Unirradiated newborn albino mouse skin	44	15	Homografts	32
Irradiated newborn albino mouse skin	10	2		
Albino mouse skin + Freund's adjuvant	11	4		
Adult albino tissues (spleen, kidney)	9	3		
Unirradiated newborn albino rat skin	11	0	Hetero-specific grafts	18
Irradiated newborn albino rat skin	11	4		
Unirradiated adult baboon skin	11	0	Hetero-generic protein	0
M. butyricum emulsion (Freund's)	13	0		

*From previous data, Cohen and Cohen, 1957.

adjuvant to the inoculum did not significantly enhance the response of the tumour to radiation. The effect of other homologous organs, one predominantly epithelial tissue (kidney, 2/5 cures), and one predominantly connective tissue (spleen, 1/4 cures) also did not differ significantly from that of skin, although these experiments were done on rather small groups of mice.

As the genetic and antigenic constitution of the inocula diverged more widely from those of the C3H mouse, the cure-rate tended to fall; skin from a different, but related species (rat), yielding 18 per cent cures in a pooled group of 22 mice, and tissue from a primate (baboon) as well as a suspension of bacteria (*M. butyricum*) yielding no cures in a pooled group of 24 mice.

DISCUSSION

The foregoing results appear to confirm the prediction that unirradiated homologous tissues and radiation-attenuated isologous tissues have similar radiosensitizing effects. The inoculation of both types of " antigen " into tumour-bearing animals amounts to the super-imposition of " equivocated information " upon the host-tumour system, and if the new information is not readily distinguished from the existing tumour signal-pattern, it will disturb the relatively tolerant host-tumour relationship. This would be expected to amplify the effects of local irradiation, and hence account for the induced radiosensitization of tumours.

It is also interesting to note that homografts have the maximum radiosensitizing capacity which is presumably associated with an optimal antigenic stimulus. On the other hand, grafts that are either more, or less, closely related immuno-

genetically to the tumour-bearing mouse give inferior results. In this respect, the variation in the cure-rate of the tumour as a function of the changing immunogenetic characteristics of the sensitizing inocula resembles that obtained in the case of isografts subjected to increasing attenuating doses of ionizing radiation (Cohen and Cohen, 1957). One might speculate that the optimal attenuating dose of radiation converts the isograft to a virtual homograft, while higher doses denature the isograft beyond this stage so that it acts like any non-specific foreign protein.

It would seem that, whatever type of antigenic stimulus is applied to this particular host-tumour relationship, it is apparently not possible to obtain cure-rates beyond a maximum of about 50 per cent at the standard test-dose of 4200 r (Cohen and Cohen, 1957). Since the LD_{50} of the non-sensitized tumour has remained consistently at 5700 r throughout this series of experiments (Cohen and Cohen, 1953; and unpublished data, 1958), the maximum degree of radiosensitization obtainable may be estimated by the ratio of these two doses. In other words, the radiosensitivity can be increased by no more than a factor of 36 per cent. This slight increase in curability seems to be a fundamental limitation of the stable C3H isologous host-tumour relationship, and it is highly improbable that any better results could be obtained by this approach in the therapy of human tumours. However, since a sensitized tumour might respond to a dose of about three-quarters of that normally required, the technique could have some limited application in clinical therapy of radio-resistant tumours.

SUMMARY

C3H mice bearing isografts of the mammary adenocarcinoma were each given a single inoculum of (1) hybrid neonatal skin from (C3H × stock albino) F_1, (F_1 × stock albino) backcross, and (C3H × F_1) backcross mice; (2) irradiated and unirradiated homologous neonatal skin from stock albino mice, homologous neonatal skin emulsified in Freund's adjuvant, and homologous spleen and kidney from adult stock albino mice; (3) irradiated and unirradiated rat neonatal skin; and (4) completely foreign proteins in the form of baboon skin and of *Mycobacterium butyricum* with Freund's adjuvant. This was followed in 2–5 weeks by treatment of the tumour *in situ* with a test dose of 4200 r, a dose level at which the expectation of cure in control mice is about 0·1 per cent. The overall cure rate in the hybrid-graft groups was 16 per cent, in the homograft groups 32 per cent, in the heterospecific-graft groups 18 per cent, and, in the heterogeneric groups there were no cures. This trend seemed to indicate that optimal radiosensitization of the tumour could be obtained where the antigenic constituents of the inocula resembled, but were neither identical with, nor too divergent from, the antigens of the tumour and the host.

The limitations of this combined immunological and radiotherapeutic attack on stable isologous and autologous host-tumour relationships are discussed.

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THE MODIFYING INFLUENCE OF DIET AND THE PHYSICAL ENVIRONMENT ON SPONTANEOUS TUMOUR FREQUENCY IN RATS

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THE unusually high frequency of phaeochromocytoma in albino Wistar strain rats bred for many years in our laboratory in Johannesburg compared with that reported from the Wistar Institute by Yeakel (1947) suggested that environmental factors among other conceivable factors were able to influence the development of spontaneous neoplasms in the albino rat (Gillman *et al.* 1953). It is known that diet can modify the speed of emergence of neoplasms induced experimentally by means of specific carcinogens (Rusch, 1944 ; Tannenbaum, 1944 ; Yamagiwa and Itchikawa, 1914 ; Berenblum, 1954). However, apart from the classical work of McCay (1942) and of Saxton *et al.* (1948) there is little information about the influence of diet on the frequency of spontaneously-occurring neoplasms in the rat. Moreover, despite its widespread use for studies on experimental cancer, there is a singular lack of statistical information about the risk to cancer of the rat living under a diversity of environmental conditions in different parts of the world.

Although it was our original purpose to examine the effects of diet and of geographical factors on the frequency of phaeochromocytoma, the present investigation has been broadened to include a statistical analysis of the kind and frequency as well as the age and sex distribution of other spontaneously-occurring neoplasms. In the presentation of the data, we shall set on record, first, the tumour frequency in terms of age and sex in 586 of our own albino rats (henceforth referred to as the GG strain) receiving the basal diet of the colony and secondly, the effects on tumour frequency of four different diets, none of which decreased the expectation of life.

Since we have repeatedly emphasized that climate and other environmental factors may influence the utilization of food (Gillman and Gilbert, 1954) the question arose as to whether albino rats obtained from two different laboratories in Europe would develop the same kinds of tumours as those to be described in the GG strain and whether the pattern of tumour frequency would persist in the migrant strains in subsequent generations, as they do in the GG strain. The results of these several experiments will reveal that whereas the frequency of some tumours remains unaffected that of others is reduced or increased by appropriate manipulation of the environment. Accordingly, attention will be drawn to the need for defining the relative contribution of genetic and environmental factors in promoting the pattern of tumour frequency in a given strain of albino rat.

MATERIAL AND METHODS

Three groups of albino rats, comprising a total of 639 male and 700 female animals, derived originally from the Wistar strain, were used in the present investigation. The first and largest group consisted of 997 (445 male and 552 female) GG strain rats, a strain which has been bred in our laboratory in Johannesburg for the last 17 years. These rats were subdivided into 5 subgroups. The control series of 586 rats (237 males and 349 females) received the basal diet of the colony (diet 1, Table I); 163 rats received diet 2, 137 rats diet 3, 84 rats diet 4 and 27 rats diet 5.

TABLE I.—*Composition of Diets*

Diet 1 (basal)—		Diet 2—		Diet 3—		Diet 4—	
Wheat flour	54	Corn starch	76	Potato	69	Casein	
Maize	13	Fat	8	Fat	8	Fat	
Fat	6	Fibrin	10	Skimmed milk powder	15	*Salts	
Vitaminized oil	2	Brewer's yeast	5	Brewer's yeast	8	Food yeast	
Raw linseed oil	2	*Salts	1	Vitamins A, E, D		Vitamins A, E, D	
Brewer's yeast	2·6	Vitamins A, E, D					
Skimmed milk powder	18·7						
*Salts	1·7						
	%		%		%		%
Carbohydrate content	60·4		78·0		65·9		3·6
Protein	15·2		12·3		14·8		81·6
Fat	11·3		8·1		8·6		10·2

* Steenbock 40.

The second group consisted of 134 rats. Of these, 85 were obtained through the courtesy of Dr. E. Saxen of the Pathological Institute of Helsinki from an animal breeding laboratory in Copenhagen and were sent to our laboratory by air. The ages of these migrant rats, which will be referred to as the Copenhagen strain, ranged from 4 to 6 weeks at the time of transfer. The remaining 49 (24 male and 25 female) constituted the first generation offspring of the Copenhagen strain migrants, born and bred in our laboratory in Johannesburg.

The third group of 208 Utrecht strain rats comprised three series, namely, (1) 94 migrant (61 male and 33 female) rats sent to Johannesburg by air as young animals (4 to 6 weeks old) from an animal breeding Institute in Utrecht, Holland, (2) 47 of the first generation and (3) 67 of the second, third and fourth generation offspring of the Utrecht migrants born and reared in our colony in Johannesburg. The Copenhagen and Utrecht strain rats, parent as well as offspring, received the basal diet of the colony, that is, the same diet as was fed to the control rats of the GG strain.

It should be mentioned in passing that the rats obtained from Copenhagen and Utrecht respectively, although originally derived from the Wistar strain, had been bred for many years in Europe and possessed slightly different physical features from our GG strain rats.

All the rats were weighed once a week during the first 3 months after weaning and thereafter once a month until they completed their natural life span. A full autopsy was performed on every rat and the entire animal preserved in formalin. The following tissues were examined histologically as a routine procedure, namely,

the adrenal, liver, spleen, pancreas, kidney, heart, lung, thyroid, thymus and genital organs (uterus, ovary, vagina and breast in the female and testis, seminal vesicle, prostate in the male). In exceptional instances, advanced autolysis prevented satisfactory histological examination. The brain, pituitary, stomach and intestines were always examined macroscopically but were prepared for microscopic examination only when any abnormality presented itself. Any suspicious lump or ulcer in other parts of the body was sectioned as a routine. In this manner, over 13,000 tissues were sectioned for routine microscopical study. At no stage was any attempt made to select for examination only those tissues in which a tumour was suspected.

Statistical method—Comparison between rats on different diets were made on the basis of comparing the percentage of rats having the same tumour, provided the percentage exceeded 10 per cent and there were not less than 15 rats in each group. If the percentage fell below 10 or the group of rats contained less than 15, the chi-square approximation to the 2×2 contingency table was used, provided there were not less than 4 cases in each cell of the contingency table.

In cases where there were less than 4 cases in each of the cells, the exact probabilities were computed. The 5 per cent significance level was adhered to for all tests.

Since the tumour rate increased with age, it was necessary to ensure that the age structure was approximately the same for the two groups being compared. This was achieved by comparing the number of rats under a specified age and, if not significant, the percentage of tumours was then compared.

In cases where no apparent sex difference could be shown for rats on the same diet with respect to the same tumour, the sexes were combined for " between diet " comparisons. No rigid rule was adopted and each case was examined on its own merits. The same techniques as described above were used for comparing the tumour incidence in rats of the Utrecht and Copenhagen strains with that of the GG strain. All comparisons in frequency rates to be described are statistically significant.

RESULTS

I. *Tumour Incidence in the GG Strain Receiving Diet* 1

The age and sex distribution at autopsy of 586 GG strain rats receiving the basal diet is shown in Table II. Six per cent of all rats died during the first year of life ; by the age of 18 months 18 per cent had died, at 24 months 50 per cent, at 30 months 92 per cent, while the remaining 8 per cent were all dead by the age of 36 months.

In the series of 586 rats, 379 (175 males and 204 females), i.e. 64 per cent, developed one or more tumours. No tumour-bearing rats were found under the age of 12 months (Fig. 1). In the 12–18 month age group, 28 of 70 rats (40 per cent) coming to autopsy were tumour-bearers, 123 of 185 (66 per cent) in the 18–24 month group, 186 of 248 (75 per cent) in the 24–30 month group, and 38 of 43 (88 per cent) in the 30–36 month age group. That is to say, the number of tumour-bearers increased progressively with the age of the rat.

No neoplasms were detected in 207 rats, i.e. 36 per cent. These non-tumour-bearers were found in all except the most advanced 34–36 month age group (Fig. 1). Thus, up to the age of 18 months, 60 per cent of all rats autopsied were non-tumour-bearers ; between 18 and 24 months, the percentage of non-tumour-

TABLE II.—*Table Showing the Age and Sex Distribution of All Rats Coming to Autopsy*

Age group (months)	GG strain										Copenhagen strain				Utrecht strain					
	Basal diet 1		Diet 2		Diet 3		Diet 4		Diet 5		Parent		Offspring		Parent		Offspring 1st generation		Offspring 2nd, 3rd and 4th generation	
	M.	F.	M.	F.	M.	F.	M.	F.	M.	F.	M.	F.	M.	F.	M.	F.	M.	F.	M.	F.
0 <6	1	0	0	7	2	4	1	0	0	0	4	2	0	2	0	2	0	0	1	1
6 <12	3	33	6	6	8	13	4	1	1	1	0	2	0	1	4	3	4	0	0	1
12 <18	12	58	10	9	11	4	8	0	1	1	3	2	0	1	12	9	2	3	3	5
18 <24	93	95	23	22	22	18	15	11	1	2	10	5	4	6	13	3	6	1	13	4
24 <30	124	124	33	24	23	28	12	21	5	3	22	18	12	11	21	10	5	11	14	11
30 <36	4	39	9	14	3	1	5	6	4	4	9	8	8	4	11	6	7	8	6	8
36 <42	0	0	0	0	0	0	0	0	1	3	0	0	0	0	0	0	0	0	0	0
Total rats	237	349	81	82	69	68	45	39	13	14	48	37	24	25	61	33	24	23	37	30

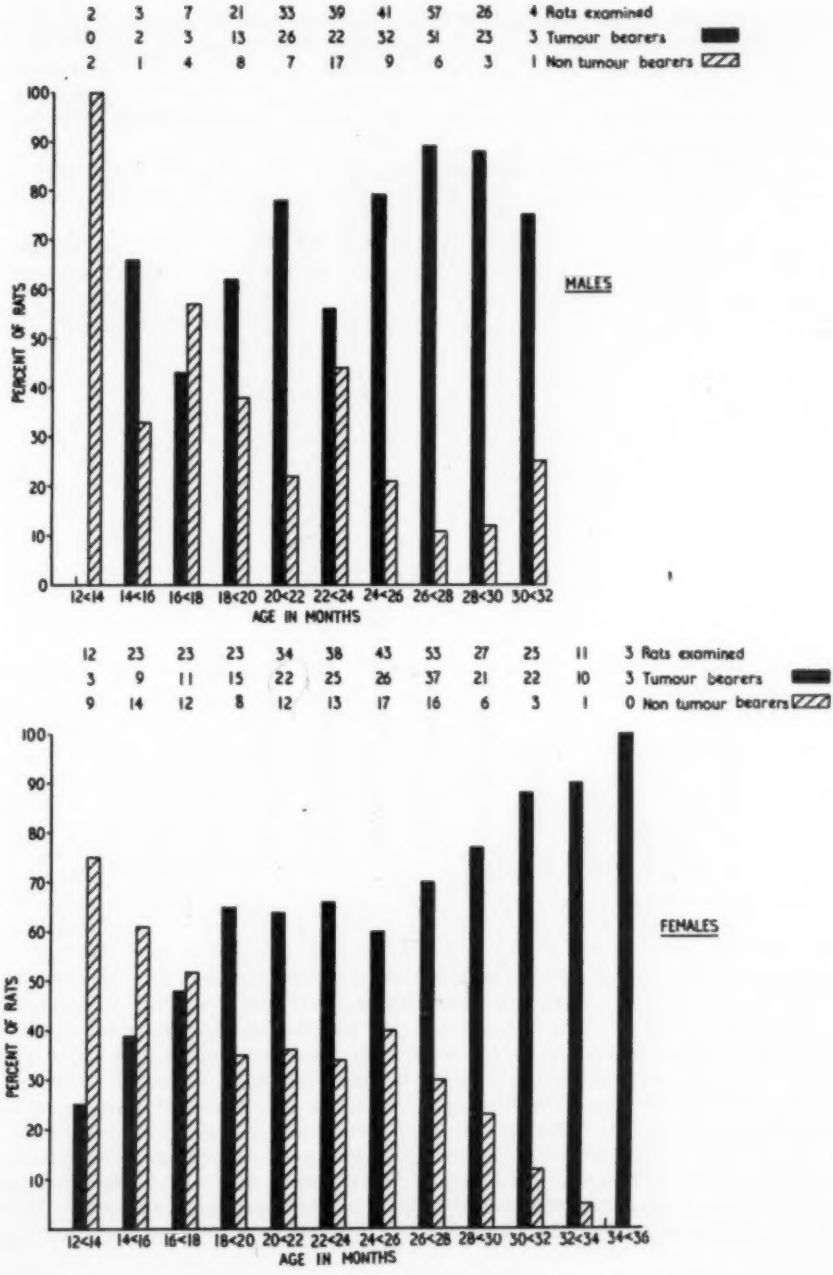

FIG. 1.—Percentage of tumour-bearers and non-tumour-bearers at successive age periods.

bearers decreased to 34 per cent (i.e. 65 of 188 rats autopsied), 25 per cent, of rats in the 24–30 month age group were non-tumour-bearing and 12 per cent in the 30–36 month age group. While age tends to render the rat susceptible to tumours, the fact that tumours do not develop in all old rats suggests that some requirement other than that dependent only on the ageing process needs to be satisfied before a tumour will develop.

A total of 520 neoplasms was encountered in 379 tumour-bearing rats, the average number of tumours per rat being 1·3 for males and 1·4 for females. The

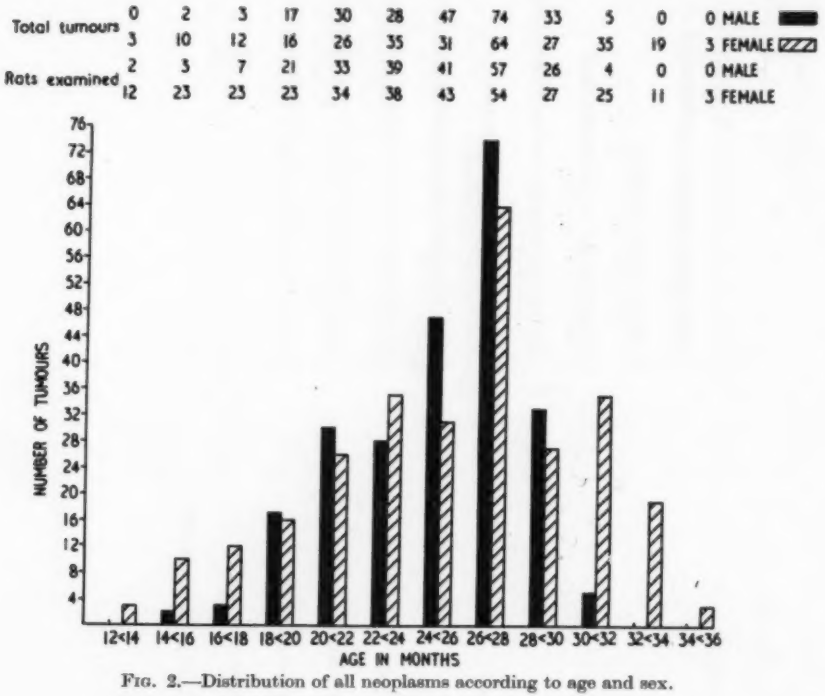

FIG. 2.—Distribution of all neoplasms according to age and sex.

majority of rats (266 of 379 or 70 per cent) bore one tumour, 23 per cent (78 of 379) two tumours, 6 per cent three tumours and less than 1 per cent four tumours.

The age and sex distribution of the 520 neoplasms are presented in Fig. 2. No tumour was observed in a rat under the age of 12 months. Two tumours were found in a total of 15 rats dying between the ages of 12 and 14 months. Thereafter the number of tumours encountered in each age group increased steadily to reach a maximum in the 26–28 month age group.

Nineteen varieties of benign and malignant tumours were observed implicating 13 different organs (Table III). In males, phaeochromocytoma, the most frequent neoplasm, was present in 62·8 per cent (137 of 218) of all rats examined. Interstitial cell tumours of the testis were detected in 18·4 per cent (42 of 228), carcinoma

TABLE III.—*Types, Order of Frequency and Percentage of Spontaneously-Occurring Neoplasms in GG Strain Rats Receiving the Basal Diet (Diet 1)*

Male				Female			
Order of frequency of tumours	Number of tissues examined	Number of tumours	Percentage of tumours	Order of frequency of tumours	Number of tissues examined	Number of tumours	Percentage of tumours
Adrenal	218	137	62·8	Adrenal	268	127	47·4
Testis	228	42	18·4	Breast	316	57	18·0
Thyroid	205	18	8·6	Pituitary	316	40	12·7
Pituitary	190	15	7·9	Thyroid	221	25	11·3
Mediastinal	232	14	6·0	Mediastinal	316	15	4·7
Pancreas:				Ovary:			
Carcinoma of islets	207	1	4·3	Thecal cell tumour	216	1	2·3
Adenoma of islets		8		Mesonephroma		2	
		9		Granulosa cell tumour		1	
Kidney:				Mesothelioma		1	
Lipofibroma	231	1	0·86			5	
Fibrosarcoma		1		Liver sarcoma	316	5	1·5
		2		Uterus:			
Liver sarcoma	231	1	0·44	Fibromyoma	219	2	1·8
				Carcinoma		2	
						4	
				Pancreas:			
				Adenoma of islets	250	2	0·8
				Submaxillary carcinoma	316	1	0·3
				Meningioma	316	1	0·3

of the thyroid in 8·6 per cent, adenoma of the pituitary in 7·9 per cent, and mediastinal tumours in 6·0 per cent. In this connection, it should be mentioned that all tumours arising from the mesothelium of the pericardium and pleura and from the reticulo-endothelial cells of the thymus and lymph nodes in the hilus of the lung were classified as mediastinal neoplasms. Noteworthy was the presence of eight islet cell tumours of the pancreas and one carcinoma of the pancreas in a total of 207 glands examined histologically.

In females, as in males, phaeochromocytoma was the most frequent neoplasm and was identified in 127 of 268 rats (47·4 per cent). Fibroadenoma of the breast (18·0 per cent) was second in order of frequency, followed by pituitary adenoma (12·7 per cent) and thyroid carcinoma (11·3 per cent). All the other neoplasms, including islet cell adenoma, carcinoma and fibromyoma of the uterus, sarcoma of the liver, submaxillary carcinoma and meningioma were present in less than 5 per cent of rats. With the exception of the carcinoma of the submaxillary gland, no tumours were found associated with the digestive tract.

Phaeochromocytoma and islet cell tumours of the pancreas showed a higher frequency in males than in females while fibroadenoma of the breast was higher amongst the females. No other sex differences in tumour frequency were shown to be statistically significant.

The age distribution of each of the neoplasms is recorded in Table IV while histograms of some of the more commonly occurring neoplasms, namely, of the adrenal, breast, pituitary, thyroid and testis are shown in Fig. 3–7. Tumours of the anterior mediastinum and fibroadenoma of the breast were the earliest to make their appearance having been observed first in rats aged 12–14 months. Phaeochromocytoma and adenoma of the pituitary were first observed at 14–16 months although, as reported previously, phaeochromocytoma can occur at the age of 11 months (Gillman, Gilbert and Spence, 1953). Liver sarcoma was first

TABLE IV.—*Age and Sex Distribution of Different Types of Spontaneously-occurring Neoplasms in GG Strain Rats (Basal Diet)*

Age group (months)	Adrenal				Thyroid				Pancreas (islet cell)				Mediastinal				Pituitary				Breast				Testis		Uterus		Ovary		Liver				Total tumours	
	RE. (M.)	T. (M.)	RE. (F.)	T. (F.)	RE. (M.)	T. (M.)	RE. (F.)	T. (F.)	RE. (M.)	T. (M.)	RE. (F.)	T. (F.)	RE. (M.)	T. (M.)	RE. (F.)	T. (F.)	RE. (M.)	T. (M.)	RE. (F.)	T. (F.)	RE. (M.)	T. (M.)	RE. (F.)	T. (F.)	RE. (M.)	T. (M.)	RE. (F.)	T. (F.)	RE. (F.)	T. (F.)	RE. (M.)	T. (M.)	RE. (F.)	T. (F.)	M.	F.
2<4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
4<6	1	0	0	0	1	0	0	0	1	0	0	0	1	0	0	0	1	0	0	0	1	0	0	0	1	0	—	—	—	—	1	0	0	0	0	0
6<8	0	0	9	0	0	0	0	0	0	0	9	0	0	0	9	0	0	0	9	0	0	0	9	0	—	—	0	0	0	0	0	0	9	0	0	0
8<10	1	0	7	0	1	0	0	0	1	0	7	0	1	0	7	0	1	0	7	0	1	0	7	0	1	0	0	0	0	0	1	0	7	0	0	0
10<12	2	0	17	0	2	0	14	0	2	0	14	0	2	0	17	0	2	0	17	0	2	0	17	0	2	0	0	0	0	0	2	0	17	0	0	0
12<14	1	0	7	0	2	0	5	0	2	0	9	0	2	0	12	2	1	0	12	0	1	0	12	1	1	0	9	0	6	0	2	0	12	0	0	3
14<16	3	2	20	8	2	0	12	0	1	0	17	0	3	0	23	0	3	0	23	2	3	0	23	0	3	0	14	0	13	0	3	0	23	0	2	10
16<18	7	3	18	5	6	0	15	0	6	0	18	0	7	0	23	2	6	0	23	0	7	0	23	3	7	0	14	0	14	0	7	0	23	2	3	12
18<20	20	10	21	7	19	0	11	0	18	0	21	0	21	2	23	2	13	1	23	3	21	0	23	3	21	3	15	0	15	1	21	0	23	0	16	17
20<22	33	19	30	15	33	2	23	1	26	0	29	1	33	2	34	1	27	2	34	5	33	0	34	1	31	4	24	0	24	0	33	1	34	1	30	26
22<24	36	19	33	19	38	0	30	4	37	0	30	0	39	1	38	1	31	3	38	4	39	0	38	5	37	5	25	0	27	0	39	0	38	2	28	35
24<26	37	26	36	14	30	3	30	2	35	5	34	0	41	1	43	3	35	4	43	3	41	0	43	8	41	8	29	1*	28	0	41	0	43	0	47	31
26<28	55	39	41	24	50	11	45	9	55	3	46	0	57	5	54	3	52	3	54	11	57	0	54	14	57	11	43	0	44	3	55	0	54	0	74	64
28<30	22	17	26	14	22	2	23	2	23	1	20	0	26	2	27	1	19	2	27	2	26	0	27	7	26	9	18	1†	15	0	26	0	27	0	33	27
30<32	4	2	24	14	3	0	20	4	4	0	18	1	3	1	25	0	3	0	25	8	3	0	25	7	4	2	15	1†	18	0	4	0	25	0	5	35
32<34	0	0	9	5	0	0	7	3	0	0	5	0	0	0	11	0	0	0	11	2	0	0	11	7	0	0	10	1*	9	1	0	0	11	0	0	19
34<36	0	0	3	2	0	0	0	0	0	0	3	0	0	0	3	0	0	0	3	0	0	0	3	1	0	0	3	0	3	0	0	0	3	0	0	3
Total		137		127		18		25		9		2		14		15		15		40		0		57		42		4		5		1		5	238	282

RE. = rats examined.
T. = tumour.
* Myoma.
† Carcinoma.

Other tumours :
1 kidney lipofibroma 26 < 28 months.
1 fibrosarcoma of kidney pelvis (male) 26 < 28 months.
1 carcinoma submaxilliary (female) 18 < 20 months.
1 meningioma (female) 20 < 22 months.

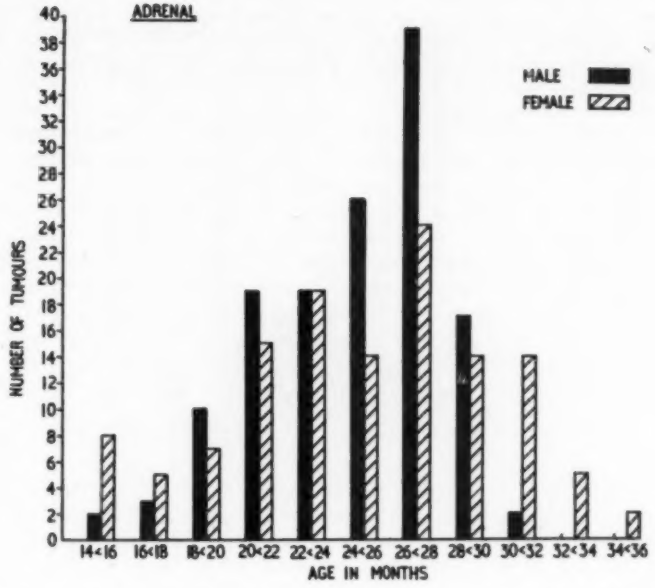

FIG. 3.—Distribution of 264 phaeochromocytoma according to age and sex.

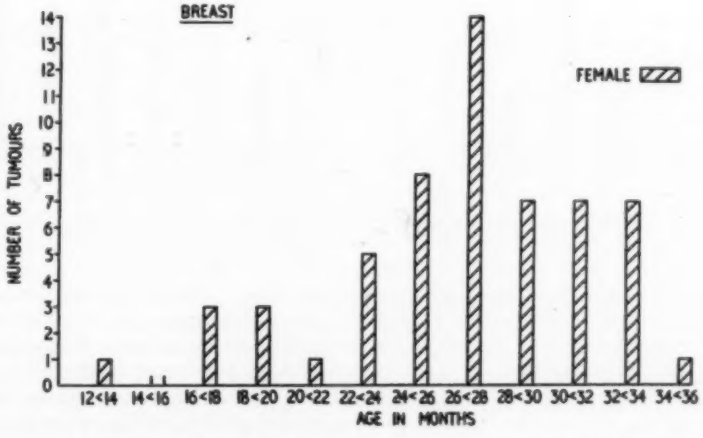

FIG. 4.—Age distribution of 57 cases of fibroadenoma of the breast.

seen at 16–18 months, ovarian and testicular neoplasms at 18–20 months and islet cell tumours of the pancreas as well as thyroid carcinoma at 20–22 months.

From the foregoing, it is evident that (1) GG strain rats show a high frequency of spontaneous neoplasms, 74 per cent of all males and 58 per cent of all females

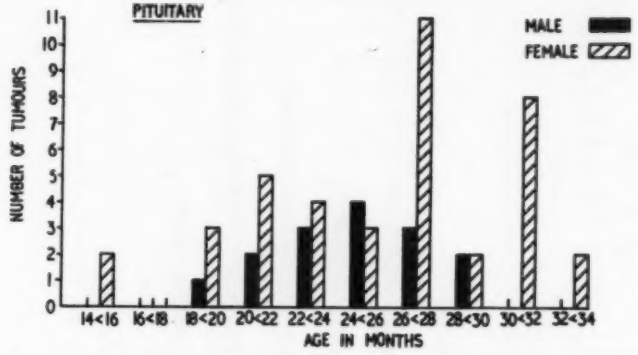

FIG. 5.—Distribution of 55 cases of pituitary adenoma according to age and sex.

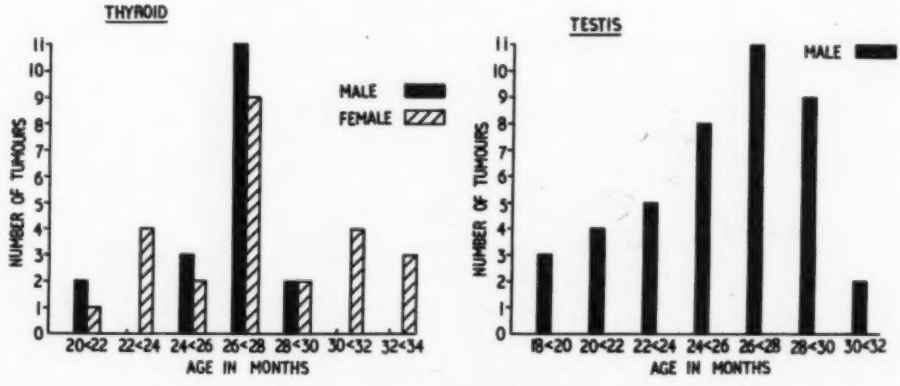

FIG. 6.—Distribution of 43 cases of thyroid carcinoma according to age and sex.
FIG. 7.—Age distribution of 42 cases of interstitial cell tumours of the testis.

being tumour-bearers when more than a year of age ; (2) neoplasms of the endocrine glands predominate in both sexes with high frequencies of phaeochromocytoma and interstitial cell tumours of the testis in males and of phaeochromocytoma and fibroadenoma of the breast in females ; (3) with the exception of mediastinal neoplasms, sarcomata were rare and (4) no neoplasms of the respiratory tract or of the epithelium of the oesophagus, stomach and intestine were found in the series of 586 rats.

II. *Effect of Diet on the Frequency Rates of Spontaneous Neoplasms*

In order to determine whether diet could modify the frequency rates of the spontaneous neoplasms described in the GG strain rats fed the basal ration of the colony (diet 1), a series of three diets was devised which, though fundamentally different from each other, were adequate to permit a steady increase in body weight and an expectation of life in accordance with that established for the GG strain in Johannesburg. Diet 2 (Table I) differed from the basal diet at least in two respects ; first, fibrin was used as the main source of animal protein in place of that derived from skimmed milk powder, and secondly, corn starch was substituted for wheat flour as a source of carbohydrate. In diet 3, carbohydrate was provided by potatoes, and animal protein by skimmed milk powder ; the amount of brewer's yeast was increased to 8 per cent (as compared with 2 per cent in diet 1) while Steenbock's salt mixture was excluded. Diet 4 contained no carbohydrate, except for the negligible amounts available in the yeast, while protein was supplied at the 77 per cent level mainly as casein. This diet could be regarded as a very high protein, carbohydrate-free, low fat diet. Diet 5 was the same as the basal diet (diet 1) except that the rats received a restricted amount of food from the time of weaning, namely, 5 g. per rat per day for the first 10 months after weaning and 7 g. per day for the remainder of their life-span. Diets 2 and 3 promoted a rate of growth as measured by body weight, similar to that of the control rats while rats receiving diets 4 and 5 showed a retarded growth rate, the average maximum weight achieved by the male rats, namely 334 g. and 264 g. respectively being significantly lower than the average maximum weight of the male controls (485 g.).

The age and sex distribution of all rats coming to autopsy in each of the above dietary groups is recorded in Table II and the percentage of rats alive at each age period in Table V. In all groups, at least 75 per cent and as many as 98 per cent of male and female rats were still alive at the age of 12 months and not less than 69 per cent (diet 3) at 18 months. In the 18–24 month age group, the mortality rate increased sharply with the result that with the exception of those receiving diet 5, the percentage of rats alive at 24 months did not exceed 69 per cent (diet 4, females) and fell as low as 37 per cent (diet 3, males). By contrast, 76 and 71 per cent of male and female rats respectively receiving diet 5 were still alive at 24 months. All rats receiving diets 1, 2, 3 and 4 were dead by the age of 36 months, whereas 15 per cent (1 out of 13) of males and 21 per cent (4 out of 14) of females receiving diet 5 survived beyond the age of 36 months but came to autopsy during the next 6-month period. Since statistical examination of the data revealed no significant differences in the expectation of life of rats receiving diets 1–5, the several dietary groups were regarded as comparable for purposes of analysis of tumour frequency rates.

Tumour-bearing rats.—The number of tumour-bearers, the total number of tumours and the rat : tumour ratio in rats receiving diets 1, 2, 3, 4 and 5 are listed in Table VI. The percentage of tumour-bearers was consistently lower in rats fed diets 2 and 3 and especially in diet 4 than in rats receiving the basal diet *ad libitum* (diet 1) or in restricted quantities (diet 5). Furthermore, the overall rat : tumour ratio was markedly reduced in rats receiving diet 4 (1 : 0·2 for males and 1 : 0·3 for females) but only slightly reduced in diets 2 and 3 as compared with control rats fed diet 1 (1 : 1·0 for males and 1 : 0·8 for females).

TABLE V.—*Expectation of Life in GG, Copenhagen and Utrecht Strains—Percentage of Rats Alive at Each Age Period*

Age period	GG strain										Copenhagen strain				Utrecht strain					
	Basal diet (1)		Diet 2		Diet 3		Diet 4		Diet 5		Parent		1st generation		Parent		1st generation		2nd and later generations	
	M.	F.	M.	F.	M.	F.	M.	F.	M.	F.	M.	F.	M.	F.	M.	F.	M.	F.	M.	F.
	(%)		(%)		(%)		(%)		(%)		(%)		(%)		(%)		(%)		(%)	
0 < 6	99	100	100	91	97	94	97	100	100	100	91	95	100	92	100	94	100	100	97	96
6 < 12	98	90	92	84	85	75	88	97	92	92	91	89	100	88	93	85	83	100	97	93
12 < 18	93	73	80	73	69	69	71	97	84	85	85	84	100	84	74	57	75	87	89	76
18 < 24	54	46	51	46	37	42	37	69	76	71	64	70	83	40	52	48	50	82	54	63
24 < 30	16	11	11	17	4	1	11	15	38	50	19	22	33	16	18	18	29	35	16	26
30 < 36	0	0	0	0	0	0	0	0	15	21	0	0	0	0	0	0	0	0	0	0
36 < 42	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

TABLE VI.—*Showing Effect of Diet on the Number of Tumour Bearers and on Rat : Tumour Ratios in the GG Strain*

Diet	Total rats		Total tumour-bearers		Percentage tumour-bearers		Number of tumours		Overall ratio of rats : tumours		Ratio of tumour-bearers : tumours		Actual number of rats with 1 or more tumours							
													1 tumour		2 tumours		3 tumours		4 tumours	
	M.	F.	M.	F.	M.	F.	M.	F.	M.	F.	M.	F.	M.	F.	M.	F.	M.	F.	M.	F.
1	237	349	175	204	74	58	238	282	1 : 1·0	1 : 0·8	1 : 1·3	1 : 1·4	120	146	47	40	8	16	0	2
2	81	82	47	34	58	41	61	47	1 : 0·7	1 : 0·6	1 : 1·3	1 : 1·4	38	25	6	5	1	4	2	0
3	69	68	38	24	56	35	45	28	1 : 0·6	1 : 0·4	1 : 1·1	1 : 1·2	32	20	6	4	0	0	0	0
4	45	39	10	12	22	31	11	13	1 : 0·2	1 : 0·3	1 : 1·1	1 : 1·1	9	11	1	1	0	0	0	0
5	13	14	9	8	69	57	10	12	1 : 0·8	1 : 0·8	1 : 1·1	1 : 1·4	8	4	1	4	0	0	0	0

As in diet 1, however, it was evident that (1) female tumour-bearers appeared to be less common than males and (2) more than 70 per cent of the tumour-bearers in each dietary group bore only one tumour. The percentage of rats carrying two tumours was highest in diet 5 (29 per cent) and lowest in diet 4 (9 per cent). The presence of 3 or of 4 neoplasms in a single rat was observed only in rats receiving diets 1 and 2 (Table VI).

From the foregoing, it is evident that diet 4 alone led to a significant reduction in the overall frequency of tumours as well as of tumour-bearing rats whereas diets 2 and 3 did not influence to any marked extent the tumour rates as compared with those observed in diet 1.

In general, it can be said that in all the dietary groups, as in the control, phaeochromocytoma was the most frequent neoplasm. However, rats fed diets 3, 4 and 5 differed from animals subsisting on diets 1 and 2 in that no sex difference was observed in the frequency of this neoplasm. The order and frequency of the other spontaneous neoplasms was variable although interstitial cell tumours of the testis and fibroadenoma of the breast in females tended to rank high in the sequence (Table VII).

By comparison with the basal diet, *diet* 2 (Table VII) led to a significant reduction in the frequency of phaeochromocytoma in both sexes, of fibroadenoma of the breast in females and of interstitial cell tumours of the testis in males. By contrast, adenoma of the pituitary in males and carcinoma of the uterus were significantly increased. Five cases of highly malignant carcinoma of the uterus were detected in a group of 58 females receiving diet 2 whereas only 2 cases of uterine carcinoma occurred in 219 female rats on the basal diet.

By comparison with the basal diet (diet 1), *diet* 3 (Table VII) promoted a significant reduction in the frequency of phaeochromocytoma in both sexes and of pituitary adenoma in females. Comparisons of the frequency of other spontaneous neoplasms disclosed no differences between rats fed diets 1 and 3.

Diet 4 (Table VII) resulted in a highly significant reduction in the frequency of phaeochromocytoma in both sexes, of fibroadenoma of the breast in females and a near significant reduction of pituitary adenoma in males as compared with rats fed diet 1. In comparison with diet 1, there was a tendency for tumour rates to be lower in rats receiving diet 4 even though the individual comparisons of tumour frequencies were not always significantly different.

When rats were fed restricted quantities of the basal diet (diet 5, Table VII), there were no significant differences in the frequencies of the neoplasms encountered as compared with rats receiving the same diet *ad libitum* (diet 1, Table I).

The differences in tumour frequency rates between rats fed diets 2, 3, 4 and those receiving the basal diet (diet 1) are summarized in Table VIII.

In Table IX attention is drawn to the first recorded appearance of each neoplasm in rats fed diets 2, 3, 4 and 5. As in the controls (diet 1), no tumour was observed under the age of one year. However, there was considerable individual variability in the time sequence of the various neoplasms. In diet 2, interstitial cell tumours of the testis which showed a low frequency were not observed before the age of 30 months as compared with 18 months in the rats fed diet 1 where the tumour rate was high. Similarly, the single case of fibroadenoma of the breast in rats receiving diet 4 was recorded in a rat aged 32 months whereas in the control rats (diet 1), this tumour was first seen at 12 months. By contrast, uterine carcinoma which showed an increased frequency in rats fed diet 2 appeared earlier (18 months)

than in the basal diet controls (24 months). The evidence suggests the existence of a possible relationship between the frequency of the neoplasm and the time of its appearance.

TABLE VII.—*Order of Frequency and Percentage of Tumours in All Necropsies of Rats Aged One Year and Over in GG Strain Rats Fed Different Diets*

Diet 2

Male				Female			
Order of frequency of tumours	Number of organs examined	Number of tumours	Percentage of tumours	Order of frequency of tumours	Number of organs examined	Number of tumours	Percentage of tumours
Adrenal	66	31	47·0	Adrenal	62	18	29·0
Thyroid	67	11	16·4	Pituitary	69	9	13·0
Pituitary	74	12	16·0	Thyroid	57	5	8·8
Testis	72	2	2·7	Uterus carcinoma	58	5	8·6
Pancreas adenoma of islets	62	1	1·6	Breast	69	4	5·8
Breast	74	1	1·3	Liver sarcoma	68	3	4·4
Kidney fibrosarcoma	74	1	1·3	Pancreas adenoma of islets	53	1	1·9
Liver sarcoma	73	1	1·3	Mediastinal	69	1	1·4
Mediastinal	74	1	1·3	Vagina carcinoma	58	1	1·7
Total	—	61	—		—	47	—

Diet 3

Male				Female			
Adrenal	58	24	41·4	Adrenal	51	19	37·3
Testis	58	8	13·8	Breast	51	4	7·8
Liver	59	5	8·5	Thyroid	42	2	4·8
Pituitary	59	4	6·8	Uterus carcinoma	50	1	2·0
Pancreas	55	2	3·6	Pituitary	50	1	2·0
Thyroid	50	2	4·0	Liver	51	1	1·9
Total	—	45	—		—	28	—

Diet 4

Male				Female			
Adrenal	45	6	13·3	Adrenal	39	6	15·4
Testis	42	4	9·5	Thyroid	36	4	11·0
Thyroid	43	1	2·3	Pituitary	38	2	5·2
				Breast	39	1	2·5
Total	—	11	—		—	13	—

Diet 5

Male				Female			
Adrenal	13	6	—	Adrenal	14	8	—
Thyroid	13	2	—	Uterus carcinoma	12	2	—
Pituitary	13	1	—	Thyroid	14	1	—
Liver sarcoma	13	1	—	Liver sarcoma	14	1	—
Total	—	10	—		—	12	—

TABLE VIII.—*Effect of Diet on Tumour Rates as Compared with Tumour Rates in Rats Fed Basal Diet (Diet 1)*

Diet	Tumour rate reduced	Tumour rate increased
2	Adrenal (male and female) Breast (female) Testis	Pituitary (male). Uterus.
3	Adrenal (male) Pituitary (female) Mediastinal (male) Liver (male)	*Nil.*
4	Adrenal (male and female) Breast (female)	*Nil.*

TABLE IX.—*Time of Appearance of Tumours*

Age group (months)	GG strain				Helsinki strain	Utrecht strain
	Diet 1	Diet 2	Diet 3	Diet 4		
12 < 14	Adrenal Breast Mediastinal	—	—	—	—	—
14 < 16	Pituitary	—	Adrenal Liver sarcoma	—	Adrenal	Adrenal
16 < 18	Liver sarcoma	Liver sarcoma	—	—	Thyroid	—
18 < 20	Testis Ovary	Adrenal Breast Uterus	Pituitary	Thyroid	—	Mediastinal
20 < 22	Thyroid Pancreas	Thyroid Pancreas Pituitary Ovary	Testis	Testis	Mediastinal Pituitary Testis	Thyroid Liver sarcoma
22 < 24	—	—	—	Adrenal	Breast	Pituitary Breast
24 < 26	Uterus	Mediastinal	Thyroid Breast	Pituitary	Pancreas	Uterus
26 < 28	—	—	Pancreas Uterus	—	—	Testis
28 < 30	—	—	—	—	Uterus	—
30 < 32	—	Testis	—	—	—	Pancreas
32 < 34	—	—	—	Breast	—	Ovary

Comment

It is evident that diet can influence the frequency of tumours in rats of the same strain and living under similar environmental conditions (Table VIII). In view of the complexity of our experimental diets, it is not possible at this stage to attribute the frequency of any tumour to the presence in or absence from the diet of any particular factor. A cereal-free, high-protein, low-fat diet (diet 4), however, gave the lowest tumour frequency rates of all the experimental diets (Table VI). Although the rats fed this diet reached a maximum weight which was well below the maximum attained by the male controls (diet 1) nevertheless, as mentioned above, their life span was not significantly different from that of rats receiving diets 1, 2, 3 or 5.

It might well be thought that the reduction in tumour frequency in rats fed diet 4 was due to interference with growth as expressed by increments of weight especially as severe caloric restriction, accompanied by marked stunting decreased the incidence and delayed the appearance of spontaneous tumours (Saxton *et al.*, 1948) and delayed the appearance of some experimentally-induced tumours induced with known carcinogens (Rusch, Johnson and Kline, 1945 ; Tannenbaum, 1942). The decrease in the frequency of spontaneous tumours in the experiments reported by Saxton *et al.* was achieved by drastic retardation of growth in such a way that the body weight was maintained constant and was only allowed to increase by amounts of 5 g. at 50-day intervals. In these circumstances, the rats, after weaning, could not gain more than 100 g. during their life-time. While extreme retardation of body growth of the order described by Saxton *et al.* may significantly alter the frequency of spontaneous tumours, a moderate degree of retardation may have unpredictable effects on tumour frequency.

The fact that the tumour frequency was significantly reduced in rats fed diet 4 and unchanged in rats fed diet 5, despite a more severe retardation of growth in the latter series, suggests that it is not only the extent but the mechanism of production of retardation which will influence the frequency of spontaneous tumours. In the case of diet 4, the weekly increments in weight during the first 3 months were on an average 20 g. below those of the controls (diet 1). At this stage, the average weight of rats fed diet 4 was 252 g. in comparison with 290 g. for the controls. Thereafter, the weight curves diverged widely ; the diet 4 fed rats increased in weight at an extremely retarded rate to reach a maximum average of 334 g. (males) while the male controls gained weight rapidly to achieve their maximum weight of 485 g. at 13 months. The gross deficiency of carbohydrate in diet 4 obliged the rats to derive calories mainly from proteins and to a lesser extent from fat. The deamination of the proteins and the excessive outpouring of urea was accompanied throughout life by a polyuria and a greatly increased consumption of water.

In the case of diet 5, the daily ration of food was not adequate to satisfy the caloric requirements. As a consequence the rats were chronically hungry and consumed their ration voraciously within a few minutes after feeding. During the early period after weaning, gains in weight occurred very slowly and at 3 months, the average weight of the males (138 g.) was more than 100 g. lower than that of rats fed diet 4. By further slow increments of weight over a period of almost 2 years, the male rats receiving a restricted food intake achieved an average maximum weight of 264 g., that is to say, an average of 70 g. less than rats on diet 4 which lacked carbohydrates. Unlike the rats fed diet 4, the restricted diet rats (diet 5) never developed polyuria or polydipsia.

It is apparent that the metabolism emphasized by rats fed diet 4 differed markedly from that of rats subsisting on the restricted diet (diet 5) and was of a kind which obviously reduced the tumour frequency in one series (diet 4) despite the better growth performance, and not in the other (diet 5). However, the fact that it was possible to alter the frequency of tumours without producing any significant differences in growth performance indicates that the metabolism promoting tumours can be uncoupled from growth beyond certain limits.

The way diet mediates its effects on the tumour-promoting mechanism still remains to be determined. Whatever the effects of diet on metabolism, these are realized in large measure by an appropriate modification in endocrine physiology.

The high frequency of endocrine tumours in our experimental rats is more than suggestive evidence that diet had profoundly altered the functional and structural integrity of the endocrine glands. Tampering directly with the endocrine glands can prevent (Gillman, Gilbert and Spence, 1955), delay (Paschkis, Cantarow and Stasney, 1948; Bielschowsky and Hall, 1953), or even accelerate the emergence of neoplasms in response to specific carcinogens (Gillman *et al.*, unpublished data). It is not surprising, therefore, to find in our experiments that the frequency of fibroadenoma of the breast, carcinoma of the uterus, interstitial cell tumours of the testis was significantly modified by particular diets (Table VIII). This does not necessarily mean that the concentration of a particular carcinogen in the diet was increased or reduced; but it may signify that the diets created a suitable endocrine context for retarding or accelerating an underlying cancerization process which may have been determined in the first instance by factors other than diet. The influence of diet on the cancerization mechanism, therefore, may be at least three-fold namely, (1) through the presence of an anti-metabolite or carcinogen, (2) through its specific effects on the vitality of particular organs (imbalance, inadequacy or excess) and (3) indirectly through its influence on the physiology of the endocrine glands.

III. *Comparison of the Frequency Rates of Spontaneous Neoplasms in Copenhagen, Utrecht and GG Strain Rats Reared in Johannesburg and Receiving the Same Diet*

The number of tumour-bearing rats and the number of tumours in the GG strain, Copenhagen, parent and first generation offspring and the Utrecht parent, first generation and second, third and fourth generation rats are presented in Table X. The percentage of tumour-bearing rats in the Copenhagen strain, parent as well as offspring, was higher both in males and in females while in the Utrecht strain it was consistently lower in males (parent and offspring) but higher in first generation, and in second, third and fourth generation females than in the GG strain.

The number of tumours per rat in the three strains under investigation was consistently higher in the Copenhagen strain, parent and offspring, than in the GG strain (Table X). In the Utrecht strain, this ratio was lower in males but higher in females as compared with the GG strain.

Examination of the data relating to the number of tumours per tumour-bearing rat disclosed that in the Copenhagen strain, unlike the GG strain, the percentage of rats with two tumours (35 per cent) was almost as great as that of rats with one tumour only (43 per cent). Furthermore, a considerable number of rats (17 per cent) bore three tumours while three rats carried four tumours (Table X). As a consequence, the number of tumours per tumour-bearing rat was higher in the Copenhagen strain (1 : 2·0 for males and 1 : 1·6 for females) than in the GG strain (1 : 1·3 for males and 1 : 1·4 for females). Similarly, in the Utrecht strain, where a relatively large percentage of females bore two or even three tumours, the number of tumours per tumour-bearing rat was consistently higher than in the GG strain females. With the exception of the first generation rats, the rat : tumour ratio in the *male* Utrecht strain was similar to that described for the GG strain males (Table X).

The first recorded appearance of the various neoplasms is presented in Table IX.

TABLE X.—*Tumour Bearers and Rat : Tumour Ratios in the GG, Copenhagen and Utrecht Strains Receiving the Same Diet (Diet 1)*

Origin of strain	Total rats		Total tumour-bearers		Percentage tumour-bearers		Number of tumours		Overall ratio of rats : tumours		Ratio of tumour-bearers : tumours		Actual number of rats with 1 or more tumours							
													1 tumour		2 tumours		3 tumours		4 tumours	
	M.	F.	M.	F.	M.	F.	M.	F.	M.	F.	M.	F.	M.	F.	M.	F.	M.	F.	M.	F.
GG (Johannesburg)	237	349	175	204	74	58	238	282	1 : 1·0	1 : 0·8	1 : 1·3	1 : 1·4	120	146	47	40	8	16	0	2
Copenhagen—																				
(Parent) .	48	37	39	31	81	84	76	51	1 : 1·6	1 : 1·4	1 : 2·0	1 : 1·6	14	16	14	11	9	3	2	1
(1st generation)	24	25	23	18	96	72	38	26	1 : 1·6	1 : 1·0	1 : 1·6	1 : 1·4	11	10	12	8	1	0	0	0
Utrecht—																				
(Parent) .	61	33	37	18	60	54	47	29	1 : 0·8	1 : 0·9	1 : 1·3	1 : 1·6	29	11	6	3	2	4	0	0
(1st generation)	24	23	13	17	54	74	22	26	1 : 0·9	1 : 1·1	1 : 1·7	1 : 1·5	7	11	3	5	3	1	0	0
(2nd, 3rd and 4th generation)	37	30	24	21	65	70	31	40	1 : 0·8	1 : 1·3	1 : 1·3	1 : 1·9	16	8	6	9	2	3	0	1

1. (a) *Copenhagen migrant strain (parent)* (Table XI)

In addition to the tumours listed for the GG strain, reticulosarcoma of the mesenteric lymph node and a fibrosarcoma of the skin were encountered in the Copenhagen strain (Table XI). As in the GG strain, phaeochromocytoma was the most frequent tumour but, unlike the GG strain, there was no sex difference in the frequency of this tumour. Indeed, apart from fibroadenoma of the breast, no significant sex differences were established for any of the neoplasms encountered in the Copenhagen strain.

By comparison with the GG strain, the Copenhagen strain showed a significant increase in the frequency of phaeochromocytoma both in males and in females, of thyroid carcinoma and mediastinal neoplasms in males, of fibroadenoma of the breast in females and a suggestively near significant increase in pituitary adenoma

TABLE XI.—*Order of Frequency and Percentage of Tumours in All Necropsies of Rats Aged One Year and Over in the Copenhagen Strain*

Copenhagen strain (parent)

Male				Female			
Order of frequency of tumours	Number of rats examined	Number of tumours	Percentage of tumours	Order of frequency of tumours	Number of rats examined	Number of tumours	Percentage of tumours
Adrenal	44	35	79·6	Adrenal	32	25	78·1
Pituitary	44	12	27·3	Breast	33	11	33·3
Thyroid	44	10	22·7	Thyroid	32	4	12·5
Mediastinal	44	10	22·7	Pituitary	33	4	12·1
Testis	42	3	7·1	Mediastinal	33	3	9·1
Pancreas (Ca)	44	3	6·8	Uterus:			
Breast	44	1	2·3	Fibromyoma	32	1	6·2
Mesenteric lymph node	44	1	2·3	Myoma		1	
Fibrosarcoma of skin	44	1	2·3	Fibroendothelioma	33	1	3·0
				Ca vagina	33	1	3·0
Total	—	76	—		—	51	—

Copenhagen (1st generation offspring)

Male				Female			
Order of frequency of tumours	Number of rats examined	Number of tumours	Percentage of tumours	Order of frequency of tumours	Number of rats examined	Number of tumours	Percentage of tumours
Adrenal	24	22	91·7	Adrenal	22	13	59·0
Pituitary	24	4	16·5	Breast	22	5	22·7
Thyroid	24	4	16·5	Thyroid	22	5	22·7
Testis	24	2	8·3	Pancreas (adenoma of islets)	21	1	4·7
Pancreas (adenoma of islets)	24	1	4·1	*Liver	22	1	4·5
Breast	24	1	4·1	*Kidney	22	1	4·5
Stomach (Ca)	24	1	4·1				
Olfactory	24	1	4·1				
Sarcoma (root of tail)	24	1	4·1				
Caecum (Ca)	24	1	4·1				
Total	—	38	—		—	26	—

* Sarcoma.

in male rats (Table XII). Interstitial cell tumours of the testis were less common (bordering significance) in the Copenhagen than in the GG strain. With this exception, there was no reduction in the frequency of any neoplasm in the Copenhagen as compared with the GG strain.

(b) *Copenhagen strain, first generation offspring* (Table XI)

The kinds of tumours and the order of their frequency in the offspring did not differ markedly from those of the parent (Table XI). However, attention is drawn to (1) the development of a carcinoma of the stomach and to a carcinoma of the caecum, (2) the absence of mediastinal tumours from males as well as from females, (3) a significantly higher frequency of phaeochromocytoma in males than in females. It will be recalled that no sex difference in the frequency of phaeochromocytoma was established in the Copenhagen parent group.

A comparison of the frequency of individual neoplasms in the Copenhagen offspring with that of the parents disclosed only one difference, namely, a decrease in mediastinal tumours in male offspring rats. The Copenhagen first generation offspring, unlike the Copenhagen parent, when compared with the GG strain, showed differences only in respect of the phaeochromocytoma which occurred more frequently in the Copenhagen male offspring than in the GG strain male rats. The previously existing differences in respect of thyroid carcinoma, fibroadenoma of the breast and interstitial cell tumours of the testis were no longer in evidence in the Copenhagen first generation offspring (Table XII).

TABLE XII.—*Comparison of Tumour Frequency Rates in Copenhagen and Utrecht Strain with GG Strain*

Diet	Tumour rate reduced	Tumour rate increased
Copenhagen (parent)	*Nil*	Adrenal (male and female). Pituitary (male). Thyroid (male). Breast (female). Mediastinal (male).
Copenhagen (1st generation offspring)	*Nil*	Adrenal (male).
Utrecht (parent)	Adrenal (male and female) Testis	Mesenteric lymph node (male).
Utrecht (1st generation offspring)	Adrenal (female)	Thyroid (male and female). Breast (female). Pituitary (near significant female).
Utrecht (2nd, 3rd and 4th generation)	Adrenal (male and female)	Pituitary (male and female). Breast (female).

It can be concluded that the frequency rates of spontaneous neoplasms in the Copenhagen first generation offspring approached more closely to those of the GG strain than did the Copenhagen parents.

2. (a) *Utrecht migrant strain (parent)* (Table XIII)

The order of frequency of the spontaneous neoplasms is listed in Table XIII. Phaeochromocytoma was the most frequent neoplasm in males, while in females

TABLE XIII.—*Order of Frequency and Percentage of Tumours in All Necropsies of Rats Aged One Year and Over in Utrecht, Parent, 1st Generation and 2nd Generation Offspring*

Parent

Male				Female			
Order of frequency of tumours	Number of tissues examined	Number of tumours	Percentage of tumours	Order of frequency of tumours	Number of tissues examined	Number of tumours	Percentage of tumours
Adrenal	55	18	32·7	Adrenal	28	7	25·0
Thyroid	57	9	15·7	Breast	28	7	25·0
Mesenteric* lymph node	57	8	14·0	Pituitary	28	5	17·8
Mediastinal	57	3	5·2	Thyroid	27	2	7·4
Pancreas (islet cell adenoma)	50	2	4·0	Mesenteric* lymph node	28	2	7·1
Testis	55	2	3·6	Liver sarcoma	28	2	7·1
Liver sarcoma	57	2	3·5	Uterus:			
Paraganglioma	57	2	3·5	(Leiomyosarcoma)	24	1	8·3
Pituitary	57	1	1·7	(Myoma)			
				Pancreas	27	1	3·7
				Ovary (thecal cell)	26	1	3·8
Total	—	47	—		—	29	—

1st Generation offspring

Male				Female			
Order of frequency of tumours	Number of tissues examined	Number of tumours	Percentage of tumours	Order of frequency of tumour	Number of tissues examined	Number of tumours	Percentage of tumours
Adrenal	20	11	55·0	Breast	23	9	39·1
Thyroid	19	4	21·0	Thyroid	23	6	26·0
Mediastinal	20	3	15·0	Pituitary	23	6	26·0
Breast	20	1	5·0	Adrenal	23	2	8·6
Testis	20	1	5·0	Pancreas (islet cell adenoma)	23	1	4·3
Mesenteric* lymph node	20	1	5·0	Liver sarcoma	23	1	4·3
Liver sarcoma	20	1	5·0	Sebaceous carcinoma of jaw	23	1	4·3
Total	—	22	—		—	26	—

2nd generation offspring

Male				Female			
Order of frequency of tumours	Number of tissues examined	Number of tumours	Percentage of tumours	Order of frequency of tumours	Number of tissues examined	Number of tumours	Percentage of tumours
Adrenal	36	14	38·0	Breast	30	13	43·3
Pituitary	35	8	23·0	Pituitary	27	11	40·7
Thyroid	34	3	8·8	Adrenal	30	6	20·0
Pancreas (islet cell adenoma)	36	2	5·5	Thyroid	27	6	22·0
Testis	37	2	5·4	Mediastinal	30	3	10·0
Mediastinal	37	1	2·7	Pancreas (islet cell adenoma)	30	1	3·3
Liver sarcoma	37	1	2·7				
Total	—	31	—		—	40	—

* Reticulosarcoma.

fibroadenoma of the breast occurred as commonly as phaeochromocytoma. Attention is also drawn to the higher frequency of mesenteric lymph node tumours (8 of 47) in male rats. With this latter exception, the kinds of tumours observed in the Utrecht strain were similar to those described in the GG strain. Sex differences in the frequency of neoplasms in the Utrecht strain were observed only in respect of pituitary adenoma which was higher in females than in males.

A comparison of the frequency rates of individual neoplasms in the Utrecht strain with that in the GG strain disclosed (1) a lower frequency of phaeochromocytoma in males and females, (2) a reduction in interstitial cell tumours of the testis, (3) a greater frequency of reticulosarcoma of the mesenteric lymph nodes in males and females, not a single case of this latter tumour having been reported in the GG strain (Table XII). It is noteworthy that reticulosarcoma comprised 13 per cent (10 of 76) of all neoplasms encountered in male and female rats of the Utrecht strain, thus ranking fourth in order of frequency.

(b) *Utrecht strain, first generation offspring* (Table XIII)

Amongst the females, the following features were noteworthy, namely, (1) fibroadenoma of the breast ranked first and constituted 39·1 per cent of all neoplasms and (2) phaeochromocytoma ranked fourth constituting only 8·6 per cent of all neoplasms (Table XIII). Amongst the male rats, attention is drawn to the absence of a pituitary adenoma and to the occurrence of only one case of reticulosarcoma of the mesenteric lymph node. A comparison of the sexes disclosed a higher frequency of phaeochromocytoma in males and of pituitary adenoma and fibroadenoma of the breast in females. In respect of the latter, the Utrecht offspring resembled the parent rats.

The frequency rates of neoplasms in male and in female rats of the Utrecht offspring did not differ significantly from that of the Utrecht parent. When the tumour frequency of the Utrecht offspring was compared with that of the GG strain, however, it was evident that (1) phaeochromocytoma was strikingly reduced in females, (2) fibroadenoma of the breast and carcinoma of the thyroid in females were increased and (3) no difference occurred in the frequency of neoplasms in male Utrecht offspring rats as compared with male GG strain rats (Table XII).

(c) *Utrecht strain, second, third and fourth generation offspring*

Since the frequency rates of spontaneous neoplasms in the Utrecht first generation offspring were not signficantly different from those of the Utrecht parent, it was decided to examine whether this pattern would be preserved in subsequent generations of these rats born and bred in our colony. Accordingly, a group of 67 rats, representing members of the second, third and fourth generation Utr cht offspring rats were grouped as a single series in order to compare the tumour frequency rate with that recorded (1) for the first generation offspring and (2) for the Utrecht parent (Table XIII).

The second, third and fourth generation rats differed in no respect from the first generation series although there was a tendency for pituitary adenoma in the males to occur more commonly than in the first generation males. When compared with the male Utrecht parent, the male second, third and fourth generation rats showed an increase in pituitary adenoma and a decrease in mesenteric lymph node tumours. No differences in tumour frequency rates were estab-

lished for the females although there was a tendency for fibroadenoma of the breast and pituitary adenoma to be more common in the second, third and fourth generation rats than in the Utrecht parent.

A comparison of the second, third and fourth generation Utrecht rats with the GG strain revealed a markedly reduced frequency of phaeochromocytoma (males and females) and an increase in fibroadenoma of the breast (females) and in pituitary adenoma (males and females) (Table VIII).

From this experiment, it can be concluded first, that the Utrecht strain even when bred to the fourth generation, maintained a persistently lower frequency of phaeochromocytoma and a higher frequency of fibroadenoma of the breast than the GG strain and secondly, that the higher frequency of reticulosarcoma of the mesenteric lymph node in the migrant Utrecht strain was not maintained in the first and subsequent generations born and bred in Johannesburg.

Comment

No statistical data are available concerning the frequency rates of spontaneous tumours of Copenhagen strain rats reared in Europe. In the case of the Utrecht strain, data are available from Basle only in respect of phaeochromocytoma and fibroadenoma of the breast (*vide infra*). Apart from the tumours mentioned, therefore, it is not possible to say whether the tumour frequency in the Copenhagen and Utrecht strain rats, as observed in Johannesburg, had departed from that occurring in these same strains in Europe.

However, the kinds and the frequencies of the tumours observed in the Copenhagen and Utrecht strains differed in several respects from the tumour pattern in rats reported previously from Europe (Guérin, 1954) and from the United States (Curtis, Bullock and Dunning, 1931 ; Saxton *et al.*, 1948). Whereas 75 per cent of all tumours in the Copenhagen and 63 per cent in the Utrecht strain originated in the endocrine glands (adrenal, thyroid, pituitary, pancreas, ovary and testis), endocrine tumours accounted for 48, 40 and 2·2 per cent of all neoplasms in the series of rats described by Saxton *et al.*, Guérin and Curtis *et al.* respectively. Tumours of the adrenal medulla were greatly emphasized in the Copenhagen and Utrecht strains (47·2 and 32·9 per cent) but were rare in the rats described in the series of Guérin (1·5 per cent), of Saxton *et al.* (1·3 per cent) and of Curtis *et al.* (0·6 per cent) (Table XIV).

Reticulosarcoma of the mesenteric lymph node constituted 16·4 and 6·1 per cent of tumours encountered respectively by Curtis *et al.* and by Guérin, while Saxton *et al.* reported a high frequency of lymphosarcoma of the lung (40 per cent.). In the Copenhagen and Utrecht parent strains, reticulosarcoma of the mesenteric lymph node comprised 0·8 and 13·1 per cent respectively of all tumours whereas in the GG strain, not a single case of lymphosarcoma occurred in a total of 520 tumours (Table XIV).

In respect of the lymphosarcoma, the Utrecht migrant strain rats undoubtedly were akin to the rats of Curtis *et al.*, but this kinship was altered in the first generation Utrecht rats when the frequency of lymphosarcoma was sharply reduced to 2·2 per cent and to 0·0 per cent in the second and subsequent generations (Table XIII). In general, it can be said that the tumour pattern of the Copenhagen and Utrecht strains, and more particularly of the first and subsequent generation offspring, resembled more closely that of the GG strain than it did the tumour pattern in albino rats reported from Europe or from the United States

TABLE XIV.—*Comparison of Frequency Rates of Endocrine Tumours and of Lymphosarcoma in Different Groups of Rats*

Type of tumour	Guérin (1954)		Curtis *et al.* (1931)		Saxton *et al.* (1948)		Utrecht strain (Parent) Johannesburg		Utrecht strain (1st generation) Johannesburg		Copenhagen strain (Parent) Johannesburg		GG strain Johannesburg	
	Number of tumours	Percentage of all tumours	Number of tumours	Percentage of all tumours	Number of tumours	Percentage of all tumours	Number of tumours	Percentage of all tumours	Number of tumours	Percentage of all tumours	Number of tumours	Percentage of all tumours	Number of tumours	Percentage of all tumours
Adrenal . .	9	1·5	3	0·6	2	1·3	25	32·9	13	27·1	60	47·2	264	50·7
Thyroid . .	89	15·5	0	0·0	0	0·0	11	14·4	10	20·8	14	11·0	43	8·3
Pituitary . .	93	16·2	0	0·0	69	46·0	6	7·9	6	12·5	16	12·6	55	10·5
Pancreas . .	1	0·2	0	0·0	0	0·0	3	3·9	1	2·1	3	2·4	11	2·1
Ovary :														
Benign . .	28	5·7	6	1·8	1	0·6	1	1·3	0	0·0	0	0·0	5	0·9
Malignant .	5													
Testis . .	3	0·5	1	0·8	1	0·6	2	2·6	1	2·1	3	2·4	42	8·1
Lymphosarcoma of mesenteric lymph node	35	6·1	74	16·4	60*	40·0	10	13·1	1	2·1	1	0·8	0	0·0
Total number of spontaneous tumours observed	567	—	452	—	150	—	76	—	48	—	127	—	520	—

* Lymphosarcoma of lung in 59 cases.

We have already indicated from experiments with the GG strain that manipulation of the diet can alter the tumour frequency. It seems to be more than a coincidence, therefore, that the Copenhagen and Utrecht strains, reared in our laboratory in Johannesburg on the same diet fed to the GG strain for one or two generations should have developed a tumour pattern similar to that of the GG strain. Apart from diet, it should be mentioned that the Utrecht and Copenhagen rats were transported from sea level in Europe to an altitude of almost 6000 feet in the Southern Hemisphere. This sudden change in altitude and in geography in itself may have influenced profoundly the metabolism of the rats. It may well be that the influence of diet on tumorigenesis in Johannesburg, such as we have described in the GG, Copenhagen and Utrecht strains, may depend not only on the qualitative and quantitative attributes of the diet but also on the simultaneously modifying effect on metabolism of local geographical and climatic factors. Perhaps the diet which led to the emergence of a tumour pattern in the Copenhagen and Utrecht strains similar to that of the GG strain in Johannesburg might induce a different pattern of tumour frequency in these same strains of rats reared in other parts of the world where climatic and geophysical factors contrasted with those operating in Johannesburg. Furthermore, the possibility still needs to be excluded that the rats transferred from Europe to Johannesburg may have been isolated from a recurring source of infection operating in early life and causally related, for example to the emergence of reticulosarcoma.

On the basis of the data available at the present time, it is suggested that until these environmental factors have been fully characterized, it is not easily possible to assess the extent to which so-called strain differences in tumour frequency are attributable to one or more specific factors in the environment or to genetic factors or possibly to a modifying influence of both. The experiments conducted on the GG, Copenhagen and Utrecht strains as well as the reports from the literature raise the question as to the criteria to be used in assessing the relative importance of environmental and genetic factors in affecting biological reactions, including the susceptibility to various kinds of spontaneous tumours. The fact that the tumour frequency in the Copenhagen first generation offspring tended to converge on a pattern similar to that recorded for the GG strain emphasizes the need for establishing the tumour frequency in rats under a variety of environments before it will be possible to know whether or not a particular tumour pattern is dependent on genetic or environmental factors.

IV. *Effect of Change of Diet and Environment on the Frequency Rates of Phaeochromocytoma and of Fibroadenoma of the Breast in GG strain and Utrecht Strain Rats*

The primary object of this experiment was to determine whether the frequency of phaeochromocytoma in Utrecht strain rats reared in Basle would be modified by rearing the same strain of rat under different conditions of diet and of the physical environment in Johannesburg and similarly, to determine whether the frequency of this tumour would be altered in GG strain rats reared in Basle as compared with those reared in Johannesburg. Accordingly, at the time of weaning, a group of Utrecht strain litters in Basle and of GG strain rats in Johannesburg were divided equally, as far as possible, with regard to numbers and sex. One half of the GG strain rats were kept in Johannesburg and the other half trans-

ported to Basle. Likewise, a group of Utrecht strain weanling rats was kept in Basle and the litter mate controls sent to Johannesburg. In this way, it was hoped to compare the frequency of phaeochromocytoma in each of the two strains of rats under the conditions operating in Johannesburg and in Basle.

As the rats came to autopsy, all the organs, including the adrenals, of the GG strain and Utrecht strain rats kept in Johannesburg were systematically preserved for histological examination, in the event that, at a later stage, it might have been possible to compare the frequency of all spontaneous neoplasms. However, in the Utrecht and GG strain rats kept in Basle, only the adrenals and the breast were routinely examined histologically ; in the case of the other organs, a measure of selection was exercised in so far as only those organs which showed macroscopically pathological changes were preserved for microscopical study. As a consequence, for purposes of *statistical* analysis, it is possible in the present study to compare only the frequency of phaeochromocytoma and of fibroadenoma of the breast in the two groups of Utrecht strain and in the two groups of GG strain rats reared simultaneously in Johannesburg and Basle. However, useful information will become available relating to the kinds of spontaneous tumours in the Utrecht strain when reared in their European environment. The Utrecht rats reared in Johannesburg received the same diet as the GG strain, namely, diet 1 (Table I) while the GG strain transported to Basle were given access to the same diet* as the Utrecht strain kept in the Ciba laboratory, Basle.

The results of the four experiments are presented in Table XV. The frequency of phaeochromocytoma and of fibroadenoma of the breast in Utrecht strain rats reared in Basle did not differ significantly from that of the litter-mate migrants in Johannesburg. Moreover, as mentioned above, the tumour frequency in the first generation Utrecht offspring, born and bred in Johannesburg, did not differ from the migrating generation, the first significant change having been observed only in the second generation in respect of reticulosarcoma of the mesenteric lymph node. It would appear, therefore, that despite the profound change in diet and in the physical environment, the Utrecht strain migrant rats in Johannesburg preserved a frequency of phaeochromocytoma and of fibroadenoma of the breast similar to that of the Utrecht strain in Basle.

The GG strain migrant rats in Basle differed from the litter mate GG strain rats in Johannesburg in two ways. First, in the migrant group, the frequency of phaeochromocytoma, both in males and in females, was reduced and secondly, the previously reported sex difference in the frequency of phaeochromocytoma in GG strain rats in Johannesburg was no longer observed in the migrant GG strain reared in Basle. No changes were recorded in the frequency of fibroadenoma of the breast either in males or in females in the migrant group as compared with the litter mate controls in Johannesburg. It is thus apparent that the GG strain showed greater susceptibility to a change in the migrant generation than did the Utrecht strain. Moreover, it is noteworthy that the migrant GG strain rats, by showing a reduction in the frequency of phaeochromocytoma, were converging on a pattern similar to that observed in the Utrecht rats reared in Basle.

The modification in the frequency of phaeochromocytoma in the migrant GG strain rats could readily be attributed to diet alone since it was shown previously that manipulation of the diet (diets 2, 3 and 4, Table VIII) consistently led to a reduction of this tumour of GG strain rats in Johannesburg. No explana-

* Wayne " Lab-blox " (Rat diet) Allied Mills, Inc., Chicago, Ill.

TABLE XV.—*Table Showing the Frequency of Phaeochromocytoma and Breast Tumours in GG Strain and in Utrecht Strain Rats Reared in Johannesburg as well as in Basle*

Type of tumour	GG strain								Utrecht strain							
	Johannesburg				Basle				Johannesburg				Basle			
	Female		Male		Female		Male		Female		Male		Female		Male	
	RE.	Tum.	RE.	Tum.	RE.	Tum.	RE.	Tum.	RE.	Tum.	RE.	Tum.	RE.	Tum.	RE.	Tum.
Phaeochromocytoma	274	122	218	136	34	7	32	9	33	7	59	16	27	2	43	9
Breast tumours	316	57	231	0	47	10	49	1	28	7	57	0	32	11	63	3

RE. = Rats examined.
Tum. = Number of tumours.

tion can be offered for the failure of the tumours of the Utrecht migrant rats to be influenced by such contrasting environments as those operating in Johannesburg and in Basle. It might be thought that this stability of the Utrecht strain was genetically determined. However, it will be recalled that the frequency of reticulosarcoma of the mesenteric lymph node in the Utrecht migrant rats was sharply reduced in the first and second generation reared in Johannesburg. The fact that the frequency of one of the tumours was modified by environment suggests that (1) the frequency of phaeochromocytoma in the Utrecht strain is not necessarily genetically determined and (2) further manipulation of the diet and possibly other environmental factors may disclose a set of conditions whereby the frequency of phaeochromocytoma may be either increased or reduced, as happened in the GG strain following manipulation of the diet.

Apart from the phaeochromocytoma and fibroadenoma of the breast which were common both to the Utrecht and GG strains, it is noteworthy that the Utrecht rats reared in Basle developed the same kinds of tumours as those already described in the GG strain (Table XVI). Tumours of the endocrine glands were common and, amongst the males, phaeochromocytoma, as in the GG strain, ranked first in order of frequency. Amongst the females, however, phaeochromocytoma was observed in only 2 of 27 (7·4 per cent) rats examined. Nevertheless, both in males and in females, this tumour occurred far more frequently in the Utrecht strain than has been reported previously in albino rats reared in Europe (Guérin, 1954).

Fibroadenoma of the breast in the Utrecht strain was common in female rats ranking first in order of frequency in contrast to GG strain females in which phaeochromocytoma ranked first. Attention is also drawn to the occurrence of tumours of the mesenteric lymph nodes both in males and in females of the Utrecht strain in Basle in contrast to GG strain migrant rats in Basle which failed to develop this tumour. It will be recalled that lymphosarcoma of the mesenteric lymph node persisted in the Utrecht migrant rats reared in Johannesburg as well as in the first generation offspring, albeit in reduced numbers.

These experiments carried out in two geographically different regions emphasize the need for defining the relative participation of environmental factors in affecting the frequency of spontaneous neoplasms in a given strain of rat. It is also suggested that whereas profound changes in the environment may modify the frequency of some neoplasms within the migrant generation, these environmental factors may

TABLE XVI.—*Order of Frequency and Percentage of Tumours in All Necropsies on Rats Reared in Basle*

Utrecht strain

Male

Order of frequency of tumours	Number of rats examined	Number of tumours	Percentage of tumours	*Minimum % of tumours
Pituitary	28	8	28·9	12·9
Adrenal	43	9	20·8	14·5
Pancreas	22	4	18·1	6·4
Thyroid	28	5	17·9	8·1
Testis	32	2	6·2	3·2
Lymph node	20	1	5·0	1·6
Breast	65	3	4·6	4·8
Gut	24	1	4·1	1·6
Liver	41	1	2·1	1·6
Lung	43	6	1·4	9·6
Stomach	—	3	—	4·8
Total	—	43	—	—

* Expressed as percentage of total possible number of male rats over the age of 1 year, namely, 62.

Female

Order of frequency of tumours	Number of rats examined	Number of tumours	Percentage of tumours	†Minimum % of tumours
Pituitary	22	8	36·3	25·0
Breast	32	11	34·3	34·3
Uterus	22	4	18·1	12·5
Lymph node	16	3	18·7	9·3
Pancreas	17	3	17·6	9·3
Thyroid	19	2	10·5	6·2
Ovary	23	2	8·7	6·2
Adrenal	27	2	7·4	6·2
Bladder	13	1	7·6	3·1
Lung	28	1	3·5	3·1
Total	—	37	—	—

† Expressed as percentage of total possible number of female rats over the age of 1 year, namely, 32.

GG strain

Male

Order of frequency of tumours	Number of rats examined	Number of tumours	Percentage of tumours	*Minimum % of tumours
Adrenal	31	9	29·0	18·7
Pituitary	12	3	25·0	6·2
Pancreas	9	2	22·2	4·1
Liver	30	1	3·3	2·1
Breast	48	1	2·1	2·1
Total	—	16	—	—

* Expressed as percentage of total number of male rats over the age of 1 year, namely, 48.

Female

Order of frequency of tumours	Number of rats examined	Number of tumours	Percentage of tumours	†Minimum % of tumours
Pituitary	21	7	33·3	14·9
Pancreas	14	4	28·5	8·5
Adrenal	34	7	20·5	14·9
Breast	47	10	21·2	21·2
Uterus	15	1	6·6	2·1
Thyroid	16	1	6·2	2·1
Lung	33	1	3·0	2·1
Total	—	31	—	—

† Expressed as percentage of total possible number of female rats over the age of 1 year, namely, 47.

need to operate for a much longer period, that is, for at least one and possibly two or more generations before the frequency of other neoplasms will be significantly affected.

SUMMARY

The results are reported of an investigation into the influence of diet and geography on the kinds of neoplasms and their frequency in three strains of albino rats.

A group of 586 GG strain rats reared in Johannesburg showed a high frequency of spontaneous neoplasms, 74 per cent of all males and 50 per cent of all females being tumour-bearers. Eighty per cent of all neoplasms were found in the endocrine

glands, phaeochromocytoma being the commonest tumour in both sexes and interstitial cell tumours of the testis and fibroadenoma of the breast ranking second in order of frequency in males and females respectively.

By radical modification in the diet, without interference with life expectation, it was possible to achieve profound changes in tumour frequency rates including, amongst others, a reduction in phaeochromocytoma and an increase in the carcinoma of the uterus in GG strain rats. A high protein, carbohydrate-free diet reduced the overall frequency of tumours as well as of tumour-bearing rats. The modifications in the frequency of endocrine tumours suggested that the diets had created at least a favourable endocrine context for retarding or accelerating an underlying cancerization process which may have been determined in the first instance by factors other than diet.

A comparison of the frequency of spontaneous neoplasms in two migrant strains of albino rats, namely, Utrecht and Copenhagen, with that of the GG strain, when maintained under similar conditions of diet and environment, disclosed significant differences in respect of some neoplasms. Some of these differences were preserved in the first and subsequent generations of migrant strains whereas others were submerged with the result that the tumour frequency converged on a pattern similar to that of the GG strain. It was suggested that until the significant environmental factors, depending on diet and possibly also on the physical attributes of the environment, such as altitude, climate etc. were identified, it would not be possible to assess the extent to which so-called strain differences in tumour frequency could be attributed to one or more specific factors in the environment or to genetic factors.

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THE EFFECT OF COPPER ACETATE ON *p*-DIMETHYLAMINO-AZOBENZENE CARCINOGENESIS IN THE RAT

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It was soon realised, following the original description by Kinosita (1940) of the development of hepatic tumours in rats treated with *p*-dimethylamino-azobenzene (DMAB), that the composition of the basal diet was of considerable importance in influencing the rate of development and yield of tumours. As a result of numerous investigations, factors in the diet which either retard or hasten tumour development have been described; these have been reviewed by Orr (1947). It must be stressed, however, that no agent has thus far been described which will completely inhibit hepatic tumour development provided the carcinogen is administered long enough.

In previous experiments (Wyatt and Howell, 1953) an unsuccessful attempt was made to produce pigmentary cirrhosis in rats maintained on a corn-grit diet supplemented with copper acetate and ferric citrate. As DMAB is an agent known to be capable of inducing cirrhosis it was decided to repeat these experiments including DMAB in the diet. The results of this experiment are now reported since it became apparent that copper acetate had a marked inhibitory effect on hepatic tumour development and cirrhosis.

MATERIALS AND METHODS

General

The experiments were carried out in two series. In Experiment A the rats were derived from entirely out-bred laboratory stock, in Experiment B, rats from a heterozygous strain were employed (Laboratory Animals Bureau Catalogue of Uniform Strains, No. 626, 1953; the Birmingham strain).

In both experiments the animals, whose age varied between 2 and 6 months at the start of the experiment, were kept in galvanised wire mesh cages, never more than 5 rats to a cage. The various diets were moistened with water to prevent the scattering of feed, and placed in galvanised troughs in the cage every morning. The amount of food given was calculated on the basis of 10 g. per rat per day. Water was available *ad lib.* In Experiment B, to check the quantity of food consumed by the rats in each cage, at certain times the residue was dried and weighed.

After the third month of treatment all the animals were examined at approximately 14 day intervals to determine the presence of palpable liver tumours. In Experiment A, animals with tumours were allowed to live their full term, or until the tumour became so large as to be incapacitating. In Experiment B the animals were usually killed as soon as, or shortly after, a tumour had become palpable.

Composition and preparation of diets

Two basic forms of diet were used: finely ground maize (obtained from a local dealer) was substituted for corn-grits, since these were unobtainable locally and rat cube powder. The latter was derived from rat cubes (Heygate and Sons, known as the Thompson diet). The Thompson diet is well balanced and has been found to maintain normal growth and nutrition. The biochemical composition of maize varies from season to season. It is very rich in nitrogen-free extract, mainly starch, and has a relatively high fat content. It never contains more than 10 per cent protein, principally zein, which is of poor biological value, and is deficient in two essential amino acids: lysine and tryptophane. Attention has been drawn to the badly balanced character of the amino acids present in zein (Gillman and Gilbert, 1954). The ergosterol, niacin, choline, riboflavin, calcium and phosphorus content is low.

Maize is of poor nutritional value for rats and when it is used as the sole source of food growth is retarded. Even when it is supplemented with lysine and tryptophane growth is still retarded and its protein value is not equivalent to that of casein (Hogan *et al.*, 1955).

The maize and cube powder were weighed and dry crystalline DMAB was added to a concentration of 0·09 per cent. Powdered copper acetate and/or ferric citrate were added in concentrations of 0·5 per cent and 2·0 per cent respectively. The diets were shaken until an intimate admixture was obtained and were stored in enamel bins. They were freshly prepared at approximately 10 day intervals.

Dietary groups

Experiment A.—Forty rats were divided into 4 groups, with equal numbers of males and females in each group. The appropriate diet for each group (Table I) was given continuously throughout the life of the animals. When the results of this experiment were analysed and it had become apparent that some factor, presumably copper acetate, was affecting the carcinogenic process it became necessary to determine whether or not in fact the copper salt was the inhibiting agent and that the inhibition was not due to the combined effect of the copper and iron salts. Furthermore, the possibility that copper interfered with the intestinal absorption of DMAB, or even combined with it in the intestinal tract, had to be considered. Accordingly, a second experiment was arranged.

TABLE I.—*Experiment A* (*Groups* 1, 2, 3 *and* 4). *Diets*

Group	Number of rats: Male	Number of rats: Female	Diet
1	5	5	CP + Fe cit + DMAB.
2	5	5	M + Fe cit + DMAB.
3	5	5	CP + Fe cit + Cu ac + DMAB.
4	5	5	M + Fe cit + Cu ac + DMAB.

CP = Cube powder, M = Maize, Fe cit = 2·0 per cent Ferric citrate, Cu ac = 0·5 per cent Copper acetate, DMAB = 0·09 per cent *p*-Dimethylaminoazobenzene.

Experiment B.—Seventy rats were divided into 7 dietary groups with equal numbers of males and females in each group. To confirm that copper acetate

was the inhibiting agent 3 dietary groups were arranged (Table II). The other dietary groups in this experiment were designed to reduce the likelihood of copper acetate interfering with DMAB absorption or of combining chemically with it in the gut. For this purpose a system of alternating feeding was devised in which the copper acetate and DMAB components of the diet were given on different days (Tables III and IV). An additional group was included to compare the protective effect of copper acetate with the protection afforded by a normal diet given on alternate days.

TABLE II.—*Experiment B* (*Groups* 5, 6 *and* 7). *Diets*

Group	Number of rats: Male	Number of rats: Female	Diet
5	5	5	M + DMAB.
6	5	5	M + DMAB + Cu ac.
7	5	5	M + DMAB + Fe cit.

M = Maize, DMAB = 0·09 per cent *p*-Dimethylaminoazobenzene, Cu ac = 0·5 per cent Copper acetate, Fe cit = 2·0 per cent Ferric citrate.

TABLE III.—*Experiment B* (*Groups* 8, 9, 10 *and* 11). *Diets : DMAB and Copper Acetate Components Given Separately*

Group	Number of rats: Male	Number of rats: Female	Diet	Number of days per week
8	5	5	M + DMAB + Fe cit	4
			M + Cu ac	3
9	5	5	M + DMAB + Fe cit	5
			M + Cu ac	2
10	5	5	M + DMAB + Fe cit	6
			M + Cu ac	1
11	5	5	M + DMAB + Fe cit	4
			CP	3

M = Maize, CP = Cube powder, Fe cit = Ferric citrate, Cu ac = Copper acetate, DMAB = 0·09 per cent *p*-Dimethylaminoazobenzene.

TABLE IV.—*Experiment B* (*Groups* 8, 9, 10 *and* 11).
To Show Days of Week on Which DMAB or Copper Acetate Was Given

Group	Monday	Tuesday	Wednesday	Thursday	Friday	Saturday	Sunday
8	Cu ac	DMAB	Cu ac	DMAB	Cu ac	DMAB	DMAB
9	,,	,,	DMAB	,,	,,	,,	,,
10	DMAB	,,	,,	,,	,,	,,	,,
11	CP	,,	CP	,,	CP	,,	,,

Basal diet maize, except where CP is indicated.

Special procedures

In Experiment A rats were subjected to liver biopsy under anaesthesia. They were biopsied in rotation at monthly intervals, from the second to the tenth months of the experiment. During Experiment B, liver function was assessed by means of the bromsulphalein excretion test.

In Experiment B the animals were weighed regularly, and it was found that all animals gained weight, although at a slower rate than normal. During the first month of the experiment animals in Group 6 showed a loss of weight which was later slowly regained. This initial loss was thought to be due to abstention from food. Animals of this group were all heavier than animals of other groups and hence were better able to undergo voluntary starvation rather than consume an unpalatable diet.

At intervals, over periods of one month the quantity of food consumed by the animals was estimated by weighing the residues daily. The results obtained are only a rough guide to the quantity of food consumed since no allowance was made for spillage or for individual variations in consumption, but it was found that the animals in the various dietary groups consumed roughly the same amounts of food.

It was essential to determine whether or not DMAB underwent any chemical alteration when it was mixed and stored with maize and copper acetate. For this purpose column chromatography was undertaken on diets which had been prepared and stored for about 2 months prior to the test. Columns were prepared from a slurry of alumina (B.D.H. activated), ligroin extracts of two diets, one consisting of maize and 0·09 per cent DMAB, the other of maize, 0·09 per cent DMAB and 0·5 per cent copper, were dropped on the columns and washed through with suitable solvents. These two columns were compared with similarly prepared columns of DMAB alone, and of a mixture of DMAB and 4-aminoazobenzene. A clear separation of the two components of the last column was obtained, DMAB flowing faster than 4-aminoazobenzene. The columns containing extracts of the diets gave only one component corresponding to DMAB. Further experiments were undertaken to determine if there was any reduction in the quantity of DMAB in the prepared stored diets. The dye was extracted from the diet and estimated colorimetrically on the day the diet was prepared and thereafter at weekly intervals for 6 weeks. No decrease in the amount of dye was detected during this period.

Post-mortem examination and histological methods

Blocks of tissue from the liver and spleen were preserved for microscopic study, together with any other tissue which showed pathological changes. At least two blocks were taken from every liver; frequently, and especially when more than one tumour was present, several blocks were taken, although no attempt was made to section every tumour.

The tissue was fixed in 4 per cent formaldehyde-saline or occasionally in Bouin's fluid. Sections were stained with Ehrlich's haematoxylin and eosin, Weigert's haematoxylin and Van Gieson, and by the periodic-acid-Schiff method using a diastase-treated control. Perls' reaction for ferric iron was carried out on all sections. Other stains used, included Gomori's reticulin method and Best's carmine stain for glycogen. Frozen sections were cut and stained for fat. Special histochemical stains for copper were also employed.

RESULTS

The first animal to develop tumours in both Experiment A and B died during the sixth month. Hence this period has been taken as the minimum induction

period and animals that died before this time have been excluded since they were not " at risk ". The results in the two experiments are detailed in Tables V and VI.

TABLE V.—*Experiment A. Details of Tumour Incidence*

	Group			
	1	2	3	4
Animals " at risk "	10	9	8	8
Months to first tumour	9	6	11	No tumour
Other animals alive at time of first tumour	9	8	5	—
Number developing tumours	8	8	4	0
Average induction time and range (months)	13·6 (9–16)	11·25 (6–16)	14·0 (11–16)	— —
Average time to death and range (Months)	13·0 (9–16)	11·0 (6–16)	12·5 (8–16)	13·5 (10–16)

Animal " at risk " = animals surviving more than 6 months.

TABLE VI.—*Experiment B. Details of Tumour Incidence*

	Group						
	5	6	7	8	9	10	11
Animals " at risk "	8	8	10	10	8	10	9
Months to first tumour	6	18	6	15	9	9	15
Other animals alive at time of first tumour	7	0	9	7	5	9	5
Number developing tumours	8	1	10	3	6	8	5
Average induction time and range (Months)	8·5 (6–10)	— —	8·5 (6–12)	15 —	11·3 (9–15)	12 (9–15)	16·2 (15–18)
Average time to death and range (Months)	8·5 (6–10)	13·3 (10–18)	8·5 (6–12)	15·1 (8–19)	10·5 (8–15)	11·4 (9–15)	14·1 (9–18)

Animals " at risk " = animals surviving more than 6 months.

Experiment A (Table V)

Group 1.—Ten animals survived 6 months, of which 8 developed tumours. The first tumour was found at 9 months, at which time 9 other animals were alive. Other tumours appeared between 9 and 16 months (average 13·6 months). The 2 rats without tumours died after 9 and 12 months' treatment.

Group 2.—Nine animals survived 6 months, of which 8 developed tumours. The first tumour was found at 6 months, at which time 8 rats were alive. Other tumours appeared between 6 and 16 months (average 11·25 months). The animal without a tumour died at 9 months as a result of liver biopsy.

Group 3.—Eight animals survived 6 months, but only 4 developed tumours. The first tumour was found at 11 months, at which time 5 rats were surviving. Other tumours appeared between 11 and 16 months (average 14 months). The 2 animals without tumours died during the eleventh and fourteenth months of treatment.

Group 4.—No animal developed a tumour in this group although 8 survived treatment for 6 months. They died between 10 and 16 months (average 13·5 months).

Comment.—In Groups 1 and 2 combined, only 3 animals died without tumours. However, there was a difference between the two groups of 2·3 months in the

average tumour induction time. This difference may indicate a protective effect of rat cube due to its greater nutritional value as compared with maize.

When copper acetate was given with the carcinogen in a maize diet (Group 4) no tumours were found despite the fact that the group survived for an average period of 13·5 months, i.e. 2·25 months longer than the corresponding group without copper (Group 2). With a cube and copper diet (Group 3), 4 of a possible 8 animals developed tumours, the average time of tumour development being 14 months. Although this is only 0·4 months longer than in the corresponding group without copper (Group 1), the first tumour in Group 3 did not appear until the eleventh month, whereas the first tumour in Group 1 appeared during the ninth month. In Group 1, furthermore, 8 animals out of a possible 10 developed tumours.

It is apparent from these results that diets containing ferric citrate and copper acetate, especially when incorporated into a maize diet, afford a considerable degree of protection against hepatic tumour development. When given in conjunction with a rat cube diet the protection is not so marked. Chemical analyses suggest that copper storage in the liver is greater in maize-fed animals than in cube-fed animals. This might be an important factor in determining the difference in tumour yield between the two basal diets.

Experiment B (Table VI)

Groups 5 *and* 7 may be considered together. In both groups the first tumours developed during the sixth month, and all the animals " at risk " developed tumours. The average time to death in both groups was 8·5 months.

Group 6.—Eight rats survived 6 months. One tumour was found during the eighteenth month in the sole survivor of the group. The other 7 animals had died in 10–15 months (average 13·3 months).

Group 8.—All 10 rats survived treatment for 6 months. Three tumours developed in the fifteenth month. The remaining 5 animals died between 16 and 19 months without tumours.

Group 9.—Eight animals survived 6 months' treatment, 6 of which subsequently developed tumours, the first being found after 9 months. The average time to death in tumour bearing animals was 11·3 months. The 2 animals without tumours died during the eighth month.

Group 10.—Ten animals survived 6 months' treatment, of these, 8 subsequently developed tumours, the first being found during the ninth month. The other tumours were found between 11 and 15 months, and the average time to death due to tumour development was 12 months.

Group 11.—Nine animals survived 6 months' treatment, of which 5 developed tumours. The first tumours, 3 in number, were found after 15 months. The other 2 tumours appeared at 18 months. The average time to death due to tumour development was 16·2 months. Animals died between the ninth and sixteenth months, but none had tumours.

Comment.—There is a difference in the time of death due to tumour development in Experiments A and B when groups comparable, except for the addition of ferric citrate, are considered. This difference arises from differences in experimental technique. In Experiment A the animals were allowed to live until moribund or until they died as a result of tumour growth. Liver biopsies

were done in strict rotation and no special effort was made to biopsy those suspected of having tumours. During Experiment B, when the animals were submitted to bromsulphalein excretion tests, careful abdominal palpation was carried out under anaesthesia. An effort was made to test all the animals suspected of having tumours as soon as they developed, and with certain exceptions they were then killed. In this way tumours were found at an earlier stage of development than in Experiment A. In addition, since there is some evidence that strains differ in their susceptibility to the carcinogen (Sugiura and Rhoads, 1941), part of the difference in average induction times may be due to the different strains of rat used in the two experiments.

In Experiment B it has been confirmed that copper acetate is a powerful tumour inhibiting agent and that ferric citrate is without effect on the carcinogenic process. In Group 6 receiving copper acetate, none of the 8 animals at risk died before the tenth month, by which time all the animals of Group 5 had developed and died with tumours. The solitary tumour in Group 6 did not appear until the eighteenth month. The average time to death for this group, excluding the animal with a tumour, was 12·7 months, i.e. 4·2 months longer than in Group 5.

The results of the alternating feeding experiments are more difficult to interpret since it is not certain how many of the effects observed were due to the reduced DMAB consumption or to the supplementary copper. Groups 8 and 11 may be compared. In both, tumours developed in several animals during the fifteenth month of treatment; excluding these animals there were 5 survivors in Group 8 and 3 survivors in Group 11. None of the survivors in Group 8 developed tumours even though 3 of them lived to the nineteenth month of treatment; but 2 of the 3 survivors in Group 11 developed tumours during the eighteenth month. Considering the nature of the basal diet of these groups, the expectation of tumour development is greater in Group 8, which received maize every day, than in Group 11, which received cube or maize on alternate days. Despite this the tumour incidence was less in the copper-treated group. It would thus appear that the inhibitory effect of copper feeding is just apparent in Group 8.

When Group 8 is compared with Groups 9 and 10 it can be seen that tumour inhibition is much less in the two latter groups. In Groups 9 and 10 the first tumours appeared during the ninth month and all the animals dying subsequently had tumours. The prolongation of the time to the first appearance of tumours in these two groups, together with the prolongation of the average induction times when compared with Group 7, appear greater than can be accounted for by the omission of DMAB from the diet for one or two days of each week and may be due in part to the addition of copper acetate to the diet.

PATHOLOGY

Biopsy material (Experiment A)

Histological assessment of the tissue obtained from 33 animals between the second and tenth months of dietary treatment allows comparisons to be made between animals of the 4 dietary groups. However, since only one specimen of liver is available from each group per month, it cannot be assumed that the changes in that one specimen are representative of the whole group at that

particular time. Nevertheless, the material does reveal differences among the dietary groups, both in severity and rate of development of lesions.

The early microscopical changes which follow the administration of DMAB have been described by Orr (1940), and Opie (1944) and a detailed description is unnecessary. In general the effect of DMAB is to cause the gradual piecemeal destruction of liver cells, at first in a zone adjacent to portal tracts and later throughout the lobule. This is followed by regenerative hyperplasia without normal lobular architecture. This may consist of intralobular disorganisation in which foci of regenerative cells within a lobule show a derangement of the normal radial pattern of liver cell cords, frequently with signs of pressure distortion of surrounding liver cell cords and displacement of the central vein of the lobule (Fig. 1). Regenerative hyperplasia may progress to nodular hyperplasia in which varying sized nodules of regenerating liver cells are formed without normal lobular architecture (Fig. 2). These nodules are often formed from the remains of several liver lobules. Concurrently with these changes, portal tracts show infiltration with chronic inflammatory cells and macrophages (Fig. 3) At first confined to portal tracts, these cells extend into adjacent parts of the liver lobule and towards other portal tracts. They are accompanied by reticulin fibres (Fig. 4) which are gradually superimposed by collagen and eventually a multilobular cirrhosis is produced (Fig. 2). Bile duct proliferation and dilatation also occurs, proliferation is first seen in the form of double rows of fusiform cells with poorly defined cell borders and large oval nuclei, poor in chromatin. These cells accompany the chronic inflammatory cells as they extend from the portal tracts (Fig. 5 and 6).

The development of cirrhosis and regenerative hyperplasia form a convenient means of assessing the liver damage. The development of cirrhosis can be arbitrarily divided into 3 grades : periportal fibrosis or incipient cirrhosis, and early and advanced cirrhosis. Periportal fibrosis (incipient cirrhosis) is a very early stage, in which the newly formed fibrous tissue is either confined to the portal tracts or at the most, thin strands of fibrous tissue extend only a little way into the adjacent parenchyma (Fig. 7). Macroscopically the liver is always smooth.

Early cirrhosis is a later stage, in which thin strands of fibrous tissue extend a considerable way around liver lobules and pseudo-lobules (Fig. 8). Slight granularity may or may not be seen on macroscopic examination.

In advanced cirrhosis the fibrous tissue is denser and the strands thicker than that described above. The strands frequently completely encircle the lobules and regeneration nodules, and the appearances resemble human multilobular, Laennec-type cirrhosis (Fig. 2). At this stage the liver is always macroscopically granular.

In Table VII is given the detailed incidence of the various grades of cirrhosis assessed on the maximum seen in any section, together with the presence or absence of regenerative hyperplasia of liver tissue in the 4 dietary groups. In the material from 8 rats of Group 4 no evidence of cirrhosis or of hyperplasia was seen. Material from a similar number of rats is available from Group 1, 5 of which showed varying grades of cirrhosis ; in 4 regenerative hyperplasia was present. In only 3 animals was cirrhosis absent. In Group 3, material was available from 9 rats ; 3 showed regenerative hyperplasia and only one showed evidence of cirrhosis. From Group 2, 8 rats were subjected to biopsy ; 6 of

them revealed regenerative hyperplasia, and 7 showed some degree of cirrhosis. In only one animal was cirrhosis absent.

TABLE VII.—*Experiment A. Number of Biopsies in Each Group Related to the Number of Animals Showing Regenerative Hyperplasia and Cirrhosis of the Liver*

Group	Number of biopsies	Regenerative hyperplasia	Degree of cirrhosis			
			Absent	Incipient	Early	Advanced
1	8	4	3	2	3	—
2	8	6	1	3	3	1
3	9	3	8	—	1	—
4	8	—	8	—	—	—

It is apparent that liver damage was considerably less in Group 4 than in the other 3 groups. In this group the histological changes appeared to be arrested, at least during the period under study, following the development of increased periportal cellularity associated with fusiform cell proliferation. Liver cell damage was nearly always confined to cells adjacent to portal tracts. Nevertheless, it must be stressed that the histological changes in this group were all of the type which follow DMAB administration.

From the biopsy material an estimate can also be made of the rate of development of hepatic damage. Thus, in Group 1 the specimen obtained at 3 months showed early cirrhosis. In the specimen at 4 months the liver showed incipient cirrhosis with regeneration and a marked tendency for fusiform cells to encircle hepatic lobules. Similar processes were observed in all the other animals studied ; a tumour was found during the ninth month.

In Group 2, at 2 months a very severe degree of liver damage was found with early cirrhosis and nodular hyperplasia. It seems most likely that the severity of the lesions was exceptional in the particular rat examined. In the following months the specimens all showed some degree of cirrhosis with regenerative hyperplasia. At 6 months a tumour was found.

It was not until the fourth month that pronounced changes were observed in Group 3 rats, even then the animal appeared to be an exception in that the liver showed early cirrhosis and nodular hyperplasia. No cirrhosis was found in this group subsequently, and further, although at 6 and 7 months intralobular disorganisation was present, specimens at 8 and 9 months showed only minimal changes.

In Group 4 it was not until the eighth month that marked changes were seen, consisting of fusiform cells and chronic inflammatory cells tending to encircle lobules.

From these observations it is apparent that hepatic injury developed and progressed rapidly in Group 2 rats, followed closely by rats from Group 1. Group 4 animals showed a very considerable retardation in the severity of the lesions and a considerable prolongation in the time required to produce them.

Histological changes in animals dying without tumours

An assessment of the changes seen in these animals is included since they supplement the observations made on the biopsy material and help to evaluate still further the protective effect of copper acetate.

In Experiments A and B combined 37 animals failed to develop tumours after surviving a minimum of 6 months' treatment with DMAB. With the exception of Groups 5 and 7, every group contained one or more animals without tumours.

The tumourless animals can be divided into 3 groups: those that did not receive copper acetate, those that received copper acetate continuously, and those that received copper acetate on certain days of the week only. The assessment of liver damage in these groups can be based on the criteria used for the assessment of the biopsy material. The detailed results are set out in Table VIII.

TABLE VIII.—*Cirrhosis in Animals Without Tumours in Experiments A and B*

Group	Animals "at risk"	Animals without tumours	Regenerative hyperplasia	Degree of cirrhosis			
				Absent	Incipient	Early	Advanced
1	10	2	2	—	—	2	—
2	9	1	1	—	1	—	—
3	8	4	—	2	2	—	—
4	8	8	4	3	4	—	1
6	8	7	5	2	3	1	1
8	10	7	5	2	2	3	—
9	8	2	2	—	2	—	—
10	10	2	2	1	1	—	—
11	9	4	1	4	—	—	—

Animals "at risk" = animals surviving more than 6 months.

Only 3 animals given DMAB without copper acetate (Groups 1 and 2) did not develop tumours. Regenerative hyperplasia associated with incipient or early cirrhosis was present in all 3.

Groups 3, 4 and 6 received DMAB and copper acetate continuously, and a total of 19 animals did not develop tumours. Regenerative hyperplasia was present in 9 of these animals; incipient cirrhosis was observed in 9, early cirrhosis in one, and advanced cirrhosis in 2. Cirrhosis was completely absent in 7. After the thirteenth month all the animals dying in these groups had developed incipient cirrhosis and intralobular disorganisation.

There were 15 animals without tumours in the remaining groups receiving DMAB and copper acetate separately. In 10 of these, regenerative hyperplasia was observed; incipient cirrhosis was present in 5 and early cirrhosis in 3. No animal showed advanced cirrhosis, and in 7, cirrhosis was completely absent.

These observations show quite clearly that the microscopical changes in the DMAB, copper-treated rats are of the same nature as those which result from DMAB alone, but there is a delay in the rate of development and progression of the lesions especially marked during the first 12 months of treatment. Many of the animals receiving DMAB and copper eventually developed varying grades of cirrhosis, the incipient type predominating. In some animals this was accompanied by regenerative hyperplasia, frequently associated with bile duct proliferation and dilatation; but by the time these changes had developed, all the animals treated with DMAB alone had developed and died with tumours. Nevertheless, the impression is gained from the histological material that had the copper-treated animals survived, and had treatment been continued long enough, some tumours would eventually have been produced. In fact one tumour did develop during the eighteenth month of treatment in a rat from Group 6.

Tumours and cirrhosis

Two of the tumours produced by DMAB are derived from bile duct epithelium, they are the bile duct cystadenoma and the cholangiocarcinoma ; a third tumour is derived from liver cells, the hepatoma. The gross and microscopic features of these tumours have been described in detail by numerous workers and since the tumours produced in the present experiments did not differ in any way from those previously described a detailed account is unnecessary. The lesion known as cholangiofibrosis also arises from bile duct epithelium and must be considered a premalignant lesion because in common with Opie (1944) and Firminger (1955) I have observed transitions between this lesion and cholangio carcinoma.

In Experiments A and B combined, a total of 61 animals developed tumours. In 41 of these animals the tumours were of 2 or more histological types, and were frequently intimately admixed. In the remaining 20 only one tumour was present in each liver, the tumour varying in histological type. This incidence of multiple tumours in the liver has been noted previously by several workers. The tumours which occurred in the animals receiving different amounts of copper in the diet did not exhibit any unusual features.

Macroscopic cirrhosis usually accompanies the appearance of DMAB induced hepatic tumours, but it is not an essential precursor (Miller *et al.*, 1941 ; Opie, 1944 ; Hoch-Ligeti, 1946). However, in the presence of cirrhosis the tumour yield is increased and the induction time decreased. In the present experiments a portion of non-tumorous liver tissue was taken for histological examination from all the animals with hepatic tumours. The degree of cirrhosis was assessed in the same manner as in the biopsy material. In the 61 animals with tumours, cirrhosis was absent in only 4 instances. Incipient cirrhosis was present in 12, early cirrhosis in 22 and advanced cirrhosis in 23. These figures show the close association between the development of hepatic tumours and the presence of cirrhosis. They can be contrasted with the incidence and degree of cirrhosis in the 37 non-tumour bearing animals in which cirrhosis was absent in 14, incipient in 15, early in 6, and advanced in 2.

Splenic changes

Splenic enlargement, developing early in the course of DMAB carcinogenesis, was described by Orr (1940). This enlargement was present before the liver had developed microscopical changes and became maximal during the third and fourth months of treatment, thereafter tending to shrink. Enlargement of the spleen was also observed by Edwards and White (1941) and Hoch-Ligeti (1946).

During Experiment B all spleens were weighed at post-mortem examination. In Groups 5 and 7 receiving DMAB with or without ferric citrate but no copper acetate, the mean splenic weight in the 2 groups was approximately equal (2·3 g. and 2·2 g. respectively), and approximately 1·0 g. heavier than the mean weight of the spleen in Group 6 (1·1 g.), which received DMAB and copper acetate. The difference in the splenic weights of the directly comparable groups, 5 and 6, was significant ($0{\cdot}02 > P > 0{\cdot}01$).

In the other part of Experiment B where the diets were alternated, Groups 9 and 10 had the largest spleens ; these groups received the smallest quantities of copper and the largest quantity of DMAB. In Groups 8 and 11 the mean

weights of the spleen were approximately equal, but the mean weight of both was greater than in Group 6.

As the spleen enlarges during DMAB treatment its normally sharp edges become rounded, the colour becomes darker; later the surface is dulled and shows slight granularity with pitting. The consistency is much firmer than normal.

Microscopically, the spleen is congested at first, later varying degrees of fibrosis develop. The fibrous trabeculae may become much more prominent and thicker than normal (Fig. 9). In some spleens fibrous tissue is formed in the Malpighian bodies which may become almost completely replaced by collagen (Fig. 10). In this type of fibrosis many Malpighian bodies are usually involved. Another variant is where fibrous tissue is present in rather localised areas in the pulp thickening the walls of the sinusoids (Fig. 11).

These gross and microscopic changes, which are essentially the same as those described by Orr (1940), are also observed in copper acetate treated rats, but the changes in these animals were always much less advanced than in those animals given DMAB without copper acetate.

No correlation was observed between the weight of the spleen and any of the following changes in the liver: liver weight, tumour type, the presence or absence of cirrhosis or regenerative hyperplasia. In certain animals splenic enlargement and congestion were observed without marked pathological change in the liver, and splenic fibrosis was observed without cirrhosis. These observations suggest that the splenic changes are not due to vascular obstruction in the liver, with consequent portal hypertension.

DISCUSSION

There are other reports in the literature dealing with the effect of copper on azo-dye carcinogenesis which show that the protection is probably independent of the type of salt used, since both copper acetate and copper sulphate have been found to be effective. Sharpless (1946) in a study of trace-elements and azo-dye carcinogenesis used low-riboflavin diets containing DMAB supplemented by 0·15, 0·3 and 0·5 per cent copper sulphate. No effect was observed on tumour incidence but the induction time was increased by 25–50 per cent as compared to that observed with the control diets. No other details are given in this short report, except that the non-malignant liver damage was always less than in the controls.

During the early stages of the present experiments Pedrero and Kozelka (1951) added copper (the salt used was not stated) to a diet containing the carcinogen 3′-methyl-4-dimethylaminoazobenzene which is structurally related to DMAB. The concentration of copper was either 0·25 or 0·5 per cent. The experiments were terminated after treatment for 6 months. The control group receiving the carcinogen without copper contained 30 animals of which 27 developed tumours. The diet containing 0·25 per cent copper was given to 20 rats and only 12 of these developed tumours. The first tumours in both these groups were found during the third month. The diet containing 0·5 per cent copper proved toxic and only 5 animals survived 3 months' treatment, but the liver of all these proved normal on macroscopic and microscopic examination. Why this level of copper proved toxic is not clear, except that the carcinogen

used is more powerful than DMAB and the basal diet was even less nutritionally adequate than maize. They make no mention of a toxic effect due to this level of copper in control animals given a similar diet without the carcinogen.

Clayton, King and Spain (1953) used copper-free diets and diets containing either 3·94 or 300 mg./kg. of copper sulphate. Both DMAB and 3′-methyl-4-dimethylaminoazobenzene were used and with both there was definite protection against tumour development when the higher concentration of copper was given. The incidence and severity of cirrhosis was also less in the copper-treated animals. Actual numbers of animals and times of tumour development are not given in this short paper.

In a recent paper King, Spain and Clayton (1957), whilst observing the inhibitory effect of copper acetate on tumour development due to 3′-methyl-4-dimethylaminoazobenzene, also noted that the addition of copper sulphate to the synthetic diet they used caused rapid destruction of the carcinogen. They also observed that their diet, which contained 79 per cent glucose and a considerable quantity of fat, frequently became rancid when copper sulphate was mixed with it. They concluded that much of the inhibitory effect of the copper salt on tumour development was due to destruction of the carcinogen. Nevertheless, they were still able to obtain inhibition of tumours when the diet was freshly prepared every day. Rancidity was never observed in any of the diets used in the present experiments. Because the diets were freshly prepared at approximately 10 day intervals and since no evidence of destruction of dye in the diets has been found over periods of 6 weeks, it is considered that destruction of the dye can be excluded as a factor in the retardation of tumour development in the present experiments.

The mechanism whereby copper exerts its protective effect must at present be a matter for conjecture. There are a number of possibilities. Groups 8, 9, 10 and 11 of Experiment B were designed to reduce the likelihood of a chemical alteration of the carcinogen under the influence of copper acetate in the gastro-intestinal tract. The results obtained from these groups are inconclusive since the tumour incidence and induction times were very different from those in the control group receiving DMAB every day. Analysis of the histological material from Groups 4 and 6 receiving copper and DMAB continuously show that the changes in the liver were similar to those which follow DMAB alone, but less severe. These observations suggest that DMAB in an active form was reaching the liver and that any chemical alteration or interference with absorption in the gut was incomplete.

Kensler, Sugiura and Rhoads (1940) showed that DMAB treatment causes reduction in the riboflavin content of the liver; Griffin and Baumann (1946) showed that the reduction of riboflavin in the liver was directly related to the carcinogenicity of the compound tested. Later Griffin and Baumann (1948) were able to show that hydrogenated coconut oil, a protective agent, maintained the riboflavin content of the liver when DMAB was included in the diet. Clayton, King and Spain (1953) reported that copper feeding, regardless of the simultaneous administration of the carcinogen, actually increased the riboflavin content of the liver. King, Spain and Clayton (1957) using diets containing 3′-methyl-4-dimethylaminoazobenzene and copper sulphate freshly prepared each day have reported that the riboflavin content of the liver did not fall so much as when the diet contained no copper. These observations suggest that the mechanism of

protection is similar to that of high protein diets and hydrogenated coconut oil, and is mediated through maintenance of the riboflavin content of the liver.

The importance of binding of the active carcinogenic derivative of DMAB to liver proteins has been stressed by Miller and Miller (1955). The major portion of the bound dye, which is found only in the liver, is associated with some of the soluble proteins. Copper has the ability to complex very readily with a wide variety of proteins, and copper salts have been described as being "protein-avid". King *et al.* (1957) estimated the total and bound dye in the liver of their copper-carcinogen treated animals and found that they did not increase as rapidly as in those animals not receiving copper. It is not inconceivable that the metal competes with the active portion of DMAB for binding to protein in the liver, and so prevents or delays the changes in the protein content of the hepatic cells which Miller and Miller (1955) envisaged as leading to tumour formation.

Finally there are certain physiological properties of copper through which the protective effect might be mediated. Copper is concerned with glucose metabolism (Keil and Nelson, 1934). It forms an essential part of butyryl-coenzyme A (Gubler, 1956) which catalyses the first step in the oxidation of short-chain fatty acids with 3–8 carbon atoms. Copper is aiso concerned in purine metabolism by the copper-containing enzyme, uricase. There is evidence that copper is required for the activation of the cytochrome reactions and energy transfer of cells. Copper is also moderately bactericidal and it is possible that ingestion of high concentrations produces an alteration in the bacterial flora of the bowel, so that vitamin synthesis in the bowel is altered and the balance upset. Vitamins of the B group are known to be of importance in hepatic tumour development, some accelerating tumour development, others retarding it.

Thus it can be seen that copper could alter azo-dye carcinogenesis by a variety of means, and since it is an essential part of many enzyme and bio-synthetic reactions it is conceivable that enzymes and reactions which involve copper may be altered during carcinogenesis and that a high copper diet modifies this. Alternatively a high copper diet and the consequent tissue storage may itself inactivate some enzyme system or systems which are involved in the metabolism of the dye and so prevent tumour development.

SUMMARY

Experiments are described which show that copper acetate has a powerful retarding effect on hepatic tumour development in rats treated with DMAB. Thus, of 16 rats which survived treatment with copper acetate and DMAB for longer than 6 months, only one animal developed a tumour which was found after 18 months' treatment. This can be contrasted with a control group receiving DMAB alone, in which 8 rats all developed tumours in an average time of 8·5 months.

An assessment of liver damage based on the development of cirrhosis and regenerative hyperplasia is described. From the analysis of liver tissue obtained by biopsy during the experiment, and from animals dying without tumours, it has been found that liver damage and cirrhosis is always much less in copper acetate-DMAB treated rats than in rats receiving DMAB alone. The spleen of the copper-treated rats also shows less damage and does not enlarge so much as in the group treated with DMAB alone.

Possible mechanisms through which the protective effect might be mediated are briefly discussed. It has been established that there is no alteration or destruction of the carcinogen when mixed and stored with copper acetate.

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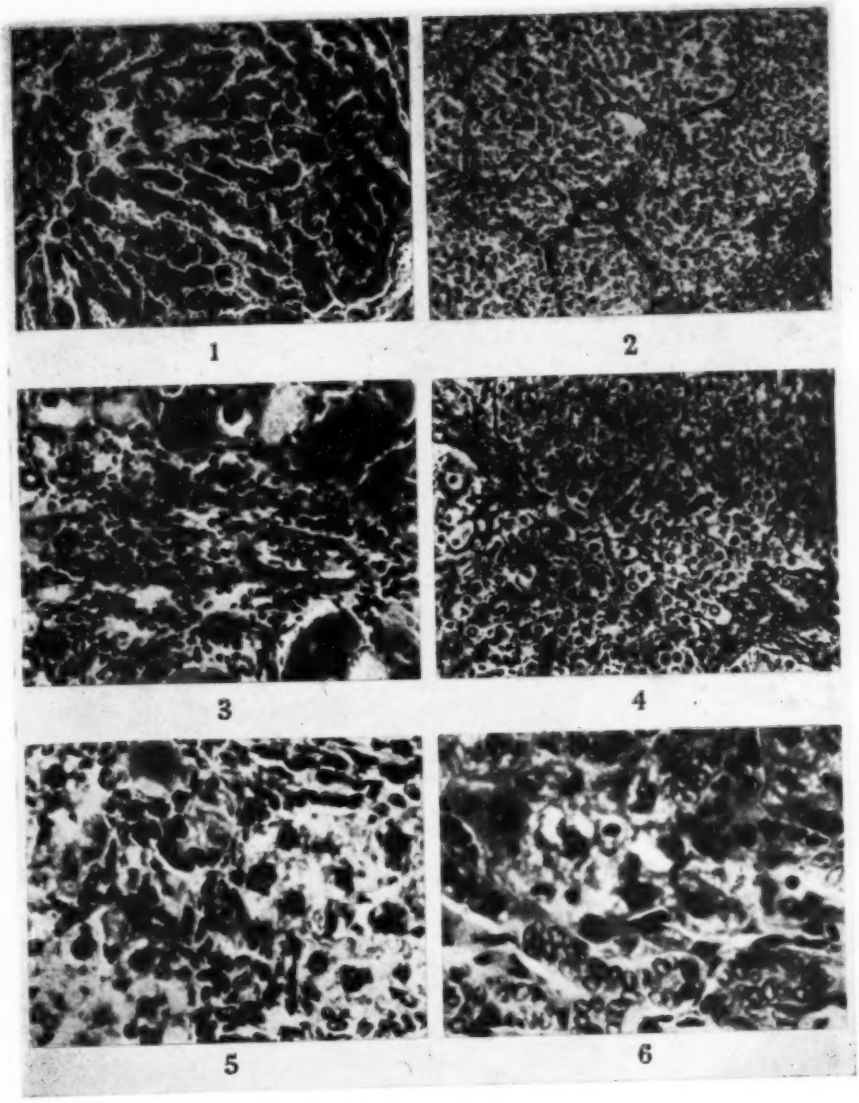
EXPLANATION OF PLATES

FIG. 1.—Intralobular disorganisation causing compression and distortion of adjacent sinusoids. H. and V.G. × 125.
FIG. 2.—Nodular hyperplasia and advanced cirrhosis. H. and V.G. × 135.
FIG. 3.—Chronic inflammatory cells and macrophages within a portal tract. Degenerative changes in periportal liver cells. H. and E. × 250.
FIG. 4.—Increased numbers of reticulin fibres radiating from portal tracts. Reticulin. × 140.
FIG. 5.—Fusiform cells extending from portal tract into adjacent parenchyma. H. and E. × 400.
FIG. 6.—Fusiform cells adjacent to a portal tract arranged in double rows. H. and E. × 460.
FIG. 7.—Periportal fibrosis (incipient cirrhosis). Delicate strands of fibrous tissue extending slightly into parenchyma. H. and V.G. × 250.
FIG. 8.—Early cirrhosis. Strands of fibrous tissue extending from portal tracts towards other portal tracts. H. and V.G. × 125.
FIG. 9.—Spleen. Increased prominence of fibrous trabeculae with marked congestion of the pulp. H. and V.G. × 125.
FIG. 10.—Spleen. Fibrosis of Malpighian body. H. and V.G. × 150.
FIG. 11.—Spleen. Fibrosis of pulp. H. and V.G. × 300.



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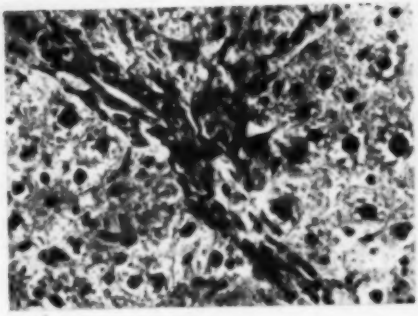

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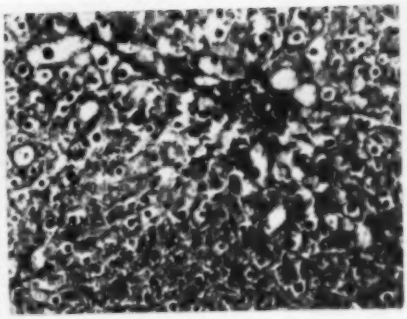

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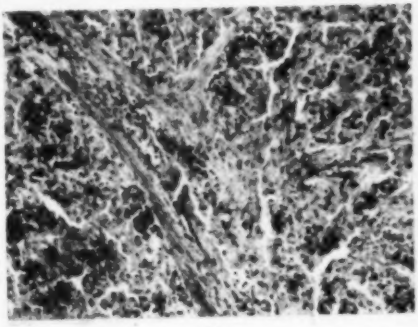

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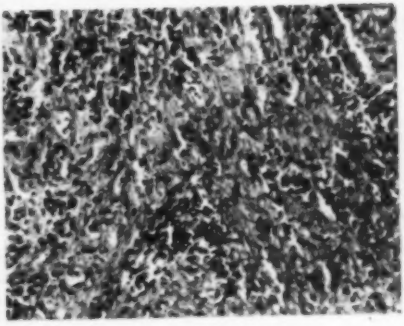

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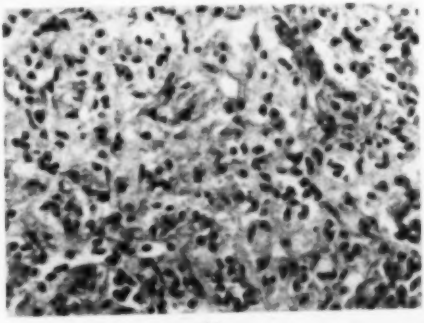

11

BROMSULPHALEIN EXCRETION IN *p*-DIMETHYLAMINOAZOBENZENE-TREATED RATS

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In a previous paper (Howell, 1958) the inhibitory effect of copper acetate on the development of hepatic tumours in rats treated with *p*-dimethylaminoazobenzene (DMAB) was described. Liver function was assessed by means of the bromsulphalein (BSP) excretion test in the hope of obtaining some biochemical measure of the rate of progression of the liver changes and to determine whether a correlation could be found between the histological changes in the liver, the type of tumour and the result of the test. It was hoped to discover whether or not functional, as well as histological differences, existed between the rats given DMAB with and without copper acetate.

The BSP test was chosen from the many available tests of liver function for a variety of reasons. It has been shown by Mateer *et al.* (1947) to be a reliable screening test in man with chronic hepatitis, cirrhosis or metastatic carcinoma, and one which could be used to measure the rate of progression or regression of these disease processes. Treatment of the rat with DMAB causes gradual destruction of liver cells and a concomitant fibrosis, and its effects can be compared with those of chronic hepatitis and cirrhosis in man in this respect. As a method of investigating liver function in experimental animals, the value of the BSP test has been demonstrated by Drill and Ivy (1944) in dogs treated with carbon tetrachloride and by Casals and Olitsky (1946) in mice subjected to various types of hepatic injury. More recently its value has again been demonstrated by Koch-Weser, Farber and Popper (1951), Waldstein, Ettinger and Giges (1957) and by Aterman and Howell (1959). The test has advantages in that the technique is fairly simple, so that a large number of animals can be tested in a short time, and also that the dye can be detected in very small quantitites of serum. Furthermore, there is no evidence that injection of the dye causes additional damage to an already damaged liver (Mateer *et al.*, 1947).

MATERIALS AND METHODS

Seventy albino rats of a heterozygous strain were used (Laboratory Animals Bureau Catalogue of Uniform Strains, No. 626, 1953; Birmingham strain). They were divided into 7 groups containing equal numbers of males and females. The dietary treatment of each of these groups (Tables I, II and III), together with the reasons for this arrangement have been given in a previous publication (Howell, 1958).

BSP tests were not undertaken before the 9th month of treatment. Tests were performed on many of the available animals during this month, again after 12 and 15 months, and at other times if it was thought that an animal had developed a hepatic tumour.

TABLE I.—*Groups 5, 6 and 7. Diets*

Group	Number of rats: Male	Number of rats: Female	Diet
5	5	5	M + DMAB.
6	5	5	M + DMAB + Cu ac.
7	5	5	M + DMAB + Fe cit.

M = Maize, DMAB = 0·09 per cent *p*- Dimethylaminoazobenzene, Cu ac = 0·5 per cent Copper acetate, Fe cit = 2·0 per cent Ferric citrate.

TABLE II.—*Groups 8, 9, 10 and 11. Diets: DMAB and Copper Acetate Components Given Separately*

Group	Number of rats: Male	Number of rats: Female	Diet	Number of days per week
8	5	5	M + DMAB + Fe cit	4
			M + Cu ac	3
9	5	5	M + DMAB + Fe cit	5
			M + Cu ac	2
10	5	5	M + DMAB + Fe cit	6
			M + Cu ac	1
11	5	5	M + DMAB + Fe cit	4
			CP	3

M = Maize, CP = Cube powder, Fe cit = Ferric citrate, Cu ac = Copper acetate, DMAB = 0·09 per cent *p*-Dimethylaminoazobenzene.

TABLE III.—*Groups 8, 9, 10 and 11. To Show Days of Week on which DMAB or Copper Acetate Was Given*

Group	Monday	Tuesday	Wednesday	Thursday	Friday	Saturday	Sunday
8	Cu ac	DMAB	Cu ac	DMAB	Cu ac	DMAB	DMAB
9	,,	,,	DMAB	,,	,,	,,	,,
10	DMAB	,,	,,	,,	,,	,,	,,
11	CP	,,	CP	,,	CP	,,	,,

Basal diet maize, except where CP is indicated.

During the test, with the animal anaesthetised, careful abdominal palpation enabled tumours to be detected at an earlier stage than was otherwise possible, and when a tumour was found the animal was usually killed immediately after completion of the test. Some animals without tumours died shortly after the completion of the test, as an effect of the test itself. Post-mortem examinations were made and blocks of liver from tumorous and non-tumorous areas were preserved. Sections were stained, and liver damage was assessed according to the methods and criteria previously described (Howell, 1958).

The technique of the BSP test has been described in detail in previous publications (Howell, 1957; Aterman and Howell, 1959). All rats were fasted over-night and then weighed to the nearest gram. A solution of BSP (G. Gurr and Sons) was injected into the exposed jugular vein, dosage being calculated on 25 mg./kg. body weight. Exactly 30 minutes after completion of the injection about 2·0 ml. of blood was withdrawn by cardiac puncture. The blood was centrifuged and the colour of the dye was developed in the serum by the addition of 10 per cent sodium

hydroxide. The amount of BSP present in the serum was estimated colorimetrically in the Unicam spectrophotometer using blanks prepared from saline and from acidified serum. It should be mentioned that certain rats, with clinically obvious tumours, were found to have icteric sera, but the results of the test in these animals were ignored since it was thought that they were likely to be fallacious.

RESULTS

Normal Rats (BSP Present in Serum 30 Minutes After Injection)

This determination was made on 15 rats with an average weight of 300 g. The mean value at 30 minutes was 0·322 mg. per 100 ml. serum (range = 0·58 to complete excretion, S.D.= 0·18). It was assumed therefore that BSP in the serum of a normal rat at 30 minutes would be very unlikely to exceed 1 mg./100 ml. serum, and values of this figure and above were regarded as abnormal; values below 1 mg./100 ml. serum were regarded as normal.

DMAB-treated Rats

Group 5. Maize and DMAB

The first tests were made after 9 months in 4 of the 5 animals surviving at this time. Three of these, including one with a palpable liver tumour, gave normal values, i.e. less than 1 mg. /100 ml. serum. The 4th animal, also with a tumour, had abnormal BSP retention. After 10 months only 2 animals remained, both with liver tumours, and abnormal BSP retention was found; they had given normal BSP values at 9 months.

Group 6. Maize, DMAB and copper acetate

All 8 available animals were tested at 10 months. None had palpable liver tumours and all had normal BSP values. After 15 months the 2 surviving rats were again tested. Neither rat had a liver tumour and both gave normal BSP values.

Group 7. Maize, DMAB and ferric citrate

Four of the 5 available rats were tested at 9 months. Three of them had palpable liver tumours, of which one had abnormal BSP retention. The tumour-free animal gave a normal result. During the 10th month an animal was tested with an abnormal result; it had a palpable liver tumour. At 12 months the sole survivor was retested, by which time a tumour had developed and the result was abnormal.

Group 8. (1) Maize, DMAB and ferric citrate, 4 days per week; (2) maize and copper acetate, 3 days per week

Three of the available 9 animals were tested at 9 months; none had liver tumours, and the result was normal in all 3. Six of the available 8 were retested at 12 months, all with normal results, none having liver tumours. Six were again tested at 15 months, all with normal results, one of the animals having a liver tumour at this time.

Group 9. (1) *Maize, DMAB and ferric citrate, 5 days per week ; (2) maize and copper acetate, 2 days per week*

Of 6 available rats, 4 were tested at 9 months. Three, including 2 with tumours, gave normal values ; the remaining animal, also with a tumour, gave an abnormal result. The 3 surviving animals in the group were retested at 11 months and 2 gave normal results ; neither having tumours ; the third had a tumour and gave an abnormal result.

Group 10. (1) *Maize, DMAB and ferric citrate, 6 days per week ; (2) maize and copper acetate, 1 day per week*

Three rats out of the 9 available were tested at 9 months. Abnormal results were obtained in 2, both of which had tumours, but the 3rd animal, also with a tumour, gave a normal result. After 12 months all 6 survivors were tested and abnormal results were obtained in 2 ; both had tumours. The 4 animals giving normal values included 2 with tumours. One of the rats tested at 12 months with a normal result was retested at 13 months, by which time a tumour had developed, and abnormal BSP retention was found.

Group 11. *Maize, DMAB and ferric citrate, 4 days per week ; rat cube, 3 days per week*

One rat at 9 months and another at 11 months were tested ; both gave normal results, neither having liver tumours. After 12 months all 6 available animals were tested, and all gave normal results. After 15 months the same animals were retested, and again all had normal results, although at this time 3 of them had liver tumours.

Correlation of BSP Test with Histology

1. *Normal BSP test*

(a) *Microscopical changes in liver of animals without tumours.*—Material is available from 6 rats, which died either at the conclusion or within one week of the test. Microscopically all showed severe liver cell damage and 3 showed varying degrees of regenerative hyperplasia. Marked periportal chronic inflammatory cell and macrophage infiltration was observed associated with proliferation of bile ducts, but in only one rat was cirrhosis seen which was early in type (Table IV).

(b) *Microscopical changes in liver of animals with tumours.*—There were 10 rats in this group, in 5 of which the tumour was confined to one or two lobes ; in the

TABLE IV.—*Rats Without Tumours Dying Shortly After BSP Test. BSP Values Correlated with Cirrhosis and Liver Weight*

Rat No.	Dietary group	BSP (mg./100 ml. serum)	Liver weight (g.)	Cirrhosis
1	11	0·72	8	−
2	11	0·62	10	−
3	10	0·61	11	−
4	8	0·71	10	−
5	6	0·66	10	++
6	6	0·53	11	−

Mean weight of liver = 10 g.
− = no cirrhosis. ++ = early cirrhosis.

remaining 5, tumours of varying size were scattered throughout all lobes of the liver. The types and combinations of tumours are set out in Table V. Sections of non-tumorous liver from these animals invariably revealed varying degrees of liver cell damage. Cirrhosis was present in 7 animals, varying from incipient to advanced in type, but was absent in the remaining 3. All 10 animals showed architectural abnormalities of the liver lobule, some showing definite regeneration nodules. All showed marked periportal chronic inflammatory cell infiltration and bile duct hyperplasia.

TABLE V.—*Normal BSP Retention Correlated with Tumour Type, Cirrhosis and Liver Weight*

Rat No.	Dietary group	BSP (mg/100 ml. serum)	Liver weight (g.)	Tumour type	Cirrhosis
1	11	C.E.	18	A	−
2	11	0·26	14	H + A	++
3	11	C.E.	18	H + A	−
4	10	„	12	A	+
5	10	„	30	A + M	++
6	9	„	12	A + C	++
7	9	0·27	11	A	++
8	8	C.E.	35	H + A	−
9	7	0·08	18	M	+++
10	5	0·68	15	H + M	+++

Mean weight of liver = 18·3 g., C.E. = Complete excretion.
H = hepatoma, A = cystadenoma, M = hepatoma + cholangiocarcinoma ,C = cholangiofibrosis.
+ = Incipient cirrhosis, ++ = early cirrhosis, +++ = advanced cirrhosis, − = no cirrhosis.

2. *Abnormal BSP test*

Microscopical changes in the liver.—Gross examination of the liver of the 11 rats with tumours and abnormal BSP retention showed that, with the exception of a single rat, the liver was diffusely involved by tumour. By this is meant that tumour nodules were found in all lobes, and whilst one lobe might show a larger tumour than the others, no definite location of a "primary" tumour could be given. The exception was an animal with a tumour confined to the right lobe of the liver. The incidence of the various types and combinations of tumours are given in Table VI. All the rats in this group showed irregular granularity of the surface of the non-tumorous parts of the liver. Microscopic examination of these areas invariably showed regenerative hyperplasia, increased cellularity of the portal tracts and bile duct proliferation. Cirrhosis was present in all these animals, being advanced in 5 and early in the remaining 5.

DISCUSSION

The results of the BSP test in DMAB-treated rats were disappointing and failed to reveal differences between animals treated with DMAB alone and those treated with DMAB and copper acetate. Furthermore, the test did not give any information regarding the severity of the pre-neoplastic changes in the liver. There were 6 animals that were tested after prolonged treatment with DMAB but which had not produced tumours. BSP excretion in these animals was entirely normal even though they all showed a severe degree of liver cell damage associated with regenerative hyperplasia, bile duct hyperplasia and in one instance early cirrhosis.

TABLE VI.—*Abnormal BSP Retention Correlated with Tumour Type Cirrhosis and Liver Weight*

Rat No.	Dietary group	BSP (mg./100 ml. serum)	Liver weight (g.)	Tumour type	Cirrhosis
1	5	1·35	10	H + A	+++
2	5	2·31	30	H	++
3	5	2·71	25	H	+++
4	7	2·80	45	M	+++
5	7	1·27	65	M + C	+++
6	7	1·37	12	H	++
7	9	3·31	65	M	++
8	9	2·15	30	H + C	++
9	10	1·27	30	M	++
10	10	1·57	40	M + A	+++
11	10	1·00	75	M + A + C	++

Mean weight of liver = 38·81 g.
H = hepatoma, A = cystadenoma, M = hepatoma + cholangiocarcinoma, C = cholangiofibrosis.
+ = Incipient cirrhosis, ++ = early cirrhosis, +++ = advanced cirrhosis.

It was not until tumours had developed that the result of the test sometimes, but not always, became abnormal. Thus 11 rats with tumours had abnormal dye retention, but in 10, also with tumours, excretion was normal; there were no major histological differences between these two groups of animals. No particular type of tumour predominated in either group, although the tumours which occur earliest in the course of DMAB carcinogenesis, e.g. cystadenomata, tended to be more frequent in the group with normal BSP excretion. Non-tumorous liver from both groups invariably showed severe damage, with necrosis of liver cells, regenerative hyperplasia, bile duct proliferation, and in all but 3 animals early or advanced cirrhosis. It is of interest that the 3 animals without cirrhosis were able to excrete the dye completely.

Although there were no major histological differences between the animals with and without abnormal dye retention there was a marked difference in the weight of the liver which could be correlated with the result of the test. The mean weight of the liver of the 10 animals with tumours, but with normal BSP excretion was 18·3 g. (Table V), but the mean weight of the liver of the 11 rats with BSP retention and tumours was 38·8 g. (Table VI). This difference is statistically significant ($0{\cdot}02 > P > 0{\cdot}01$). The tumours in the former group were smaller, more "focal" in distribution and were more often confined to one lobe of the liver than those in the latter group. The weight of the liver is a function of the amount of tumour present and the amount of destruction of normal liver parenchyma. The difference in liver size was also reflected in the ease with which a diagnosis of hepatic tumour development was made. The rats in the group with the heavier livers had obvious tumours, whereas the tumours in the other group were only found when the animals were anaesthetised.

Several points of interest arise from this study. Even in the presence of much chronic liver damage, associated with regenerative hyperplasia and the development of cirrhosis the ability of the liver to excrete BSP is unimpaired for a considerable time. Even when a tumour develops it is not until by growth and replacement of a large volume of liver tissue by tumour that the test becomes abnormal. It is possible that abnormal excretion is dependent upon the ratio of tumour to non-neoplastic liver in that this may determine the proportion of the

blood supply shunted away from functional liver tissue to non-functional tumour. However, even the presence of quite large tumours does not necessarily mean that BSP excretion will be abnormal since 5 rats with tumours, including 2 with livers weighing 30 g. or more were able to excrete the dye completely (Table V).

The reason why this test remains normal for such a considerable time is not clear. In the initial stages of the experiment considerable doubt was felt about the validity of the test because of the normal results obtained. A study was therefore undertaken of BSP excretion in 15 hypothyroid rats in which the main pathological feature was fatty change in the liver. BSP excretion was impaired in many of these animals until thyroid extract was given, when it became normal; this was followed by renewed impairment when thyroid extract was discontinued (Aterman and Howell, 1959). Hence it was concluded that the method of testing and the calculation of results of BSP excretion were reliable.

The value of liver function tests in human hepatic cancer is disputed. Berman (1951) in his monograph states that most liver function tests devised thus far are of doubtful value. Spellberg (1954) in his book states "that liver function tests are variable and depend to a great extent on an underlying cirrhosis", but also "that the BSP is likely to be increased". Sherlock (1958) in her text-book states that the BSP test is positive in hepatic cancer. Spatt and Grayzel (1948), Schupback and Chappell (1952), Galluzi *et al.* (1953) and Bersohn (1957) all found abnormal BSP excretion in their cases of primary hepatic cancer. But Holley and Pierson (1948) concluded that liver function tests as a rule were disappointing as a method of diagnosis of primary carcinoma of the liver, and that damage must be extensive before any appreciable change can be detected in the function tests. In cases of secondary carcinoma involving the liver there is greater uniformity of opinion and several workers have agreed that the BSP test is of value in diagnosis, e.g. Paulson and Wyler (1942), Thomas and Zimmerman (1952), Shay and Siplet (1954) and Sherlock (1958) and it has been suggested that the results are abnormal because of interference with blood supply caused by the secondary deposits. However, this does not explain the discrepancy of the results in primary carcinoma since the cirrhosis usually associated with this condition would be expected to cause greater interference with normal blood supply.

The clinical diagnosis of primary hepatic cancer in man is notoriously difficult and usually when the diagnosis is made and liver function tests have been undertaken the tumour is large and much destruction of liver tissue has occurred. It is concluded that there is some correlation between the results of BSP excretion in man and in the experimental animals in that the result of the test depends to a great extent on the size of the tumour and on the amount of tumour replacement of liver parenchyma. It is also concluded that the BSP test is of little value in assessing functional impairment of the rat's liver due to DMAB in the pre-neoplastic stages.

SUMMARY

Liver function has been assessed by means of the BSP excretion test in rats treated with DMAB. The results of the test, regardless of the severity of the liver damage, were entirely normal until a tumour had developed. Ten rats with tumours had normal BSP excretion and 11 rats, also with tumours, had abnormal BSP excretion. Abnormal BSP excretion is independent of tumour type and underlying liver damage, but apparently depends upon the size of tumour and

the degree of replacement by tumour of liver tissue. It is suggested that these results conform with those obtained in human cases of primary hepatic cancer.

I am indebted to Professor J. W. Orr and Dr. D. Hamer for valuable help and advice throughout this investigation. I am also indebted to the Birmingham Branch of the British Empire Cancer Campaign and to the Medical Research Fund of the United Birmingham Hospitals for support of this work.

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EXAMINATION OF TURBAN TUMOUR FOR CUTANEOUS CARCINOGENS

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AN Anglo-Indian woman, aged 58 years, suffering from turban tumour (cylindroma) had many large tumours on the body as well as the scalp. All the larger tumours were excised surgically and skin grafts were applied to the head where surgical " scalping " down to the pericranium was necessary to remove the turban tumour.

The histology of these benign basal celled epidermal growths is similar to benign epithelioma adenoides cysticum (Brooke's disease) and to the malignant basal celled epithelioma (rodent ulcer). The epidermis is little changed except by stretching over the tumour. In the dermis there are spherical, oval or irregular masses of cells. The cells are round or oval and take up the basophil stain readily, especially the nuclei. Within the cell masses are areas of pink staining hyaline-like material giving the appearance of cysts as in epithelioma adenoides cysticum. At the periphery of the cell masses the cells are arranged in a pallisade manner and stain rather more deeply than those in the centre, this is also seen in the malignant basal celled epithelioma and in Brooke's disease. A feature of the histology peculiar to turban tumours is a limiting membrane round each clump of cells which is composed of amorphous eosinophilic material similar to that composing the so-called cysts (Fig. 1). Because of their large number, size and histological similarity of the tumours in this case to benign and malignant basal celled growths they seemed to be a good source of material from which to make extracts for experiments in cutaneous carcinogenesis.

Extracts of the tumours in acetone and benzene were prepared and painted on to the epidermis of mice, twice a week for 10 months, to test for the presence of carcinogens. A further experiment was then undertaken to ascertain if the presence of co-carcinogens could be demonstrated in the extracts. This was done by exposing 20 of the extract painted mice, and 20 of the control mice from the first experiment, to large doses of ultra-violet rays for a period of 8 months. Ultra-violet irradiation of the skin will produce malignant skin tumours, and it was thought that the action of this physical agent might reveal the presence of some sensitising factor or co-carcinogen in the tumour extracts prepared. If more tumours occurred in the extract painted animals, or if the latent period of the appearance of the growths was less in these animals, then the presence of a co-carcinogen in the extracts was likely.

Extracts of non-cancerous human tissue have previously produced malignant skin tumours by painting on to the mouse's skin. Des Ligneris (1940) prepared petroleum ether extracts of cancerous and also non-cancerous livers of the Bantu native (South African), in whom primary cancer of the liver is common, and painted

these on to the skins of mice which developed benign and malignant tumours; extracts of non-cancerous European livers produced no tumours. Hieger (1940) was unable to produce malignant skin tumours when he repeated the experiment with a benzene extract of non-cancerous Bantu livers. Malignant skin tumours have occurred at the site of subcutaneous injections of extracts of non-cancerous livers of persons dying of carcinoma (Shabad, 1937; Steiner, 1942).

FIRST EXPERIMENT

Preparation of tumour extracts

Twelve tumours were removed from the trunk and vulva of my patient. Under aseptic conditions the skin covering the tumours was incised and the tumours shelled out and separated from fat and subcutaneous tissues.

The tumours were lobular, avascular and a pale yellow colour. The consistency was of soft indiarubber. The total weight of the tumours was 342 g. The whole of this material was minced through an ordinary culinary mincing machine, which had been sterilised in an autoclave. The mince was divided into two equal portions of 150 g.

One portion of the material was put into a sterile mortar with approximately half its volume of washed and incinerated sand. 100 c.c. of acetone (British Drug Houses Analytical Reagent) was then added. There was an immediate effervescence and the tissue became paler in colour due to fixing. After grinding in the mortar for 10 minutes a further 100 c.c. of acetone was added and followed by 10 minutes' grinding. A further 100 c.c. was added and the whole thoroughly mixed for a further 10 minutes. The supernatant fluid was filtered through a coarse filter paper. 236 c.c. of filtrate was obtained. which was straw coloured and slightly opalescent. This was then refiltered by suction through a " technico " filter using a Ford Sterimat anti-bacterial pad. 200 c.c., labelled Extract A, were placed in a sterile glass vessel and the top sealed with sterile cotton wool, and placed in the refrigerator at 32° F.

The other half of the material was treated in exactly the same way with benzene (British Drug Houses Analytical Reagent). There was no effervescence when it was added to the mince nor any obvious change in the benzene such as an opalescence seen in the acetone extract. 200 c.c. were labelled Extract C, and placed in the refrigerator in a sterile container as before.

In order that sufficient of each extract would be available to paint 45 mice twice weekly for a year, 200 c.c. of acetone and benzene respectively were added to the extracts, making a total of 400 c.c. of each.

Physical examination of extracts

1. *Absorption spectra.*—The absorption spectra of both extracts were non-specific and the absorption observed was probably due to extracted fat. There were no bands which could be attributed to known carcinogenic substances or metabolic products from them.

2. *Fluorescent spectra.*—The acetone extract gave a wide band between 4000 and 4500 Å; there was no discrete fluorescence which could be attributed to carcinogenic hydrocarbons. The benzene extract gave no fluorescence.

3. *Wood's light examination.*—The extracts were examined under Wood's light and the acetone extract gave a light violet-blue fluorescence. It was found

later that the areas on the mice painted with the acetone extract gave the same violet-blue fluorescence.

It was surprising that there was no fluorescence in the benzene extract, because many steroid substances which fluoresce were likely to have been extracted from the tumour tissue by the benzene.

4. *Freezing temperature.*—The lowest temperature which could be produced in the refrigerator in which the extracts were kept was 22° F. and neither the acetone nor acetone extract froze at this temperature. The benzene extract froze at 32° F. and the benzene at 30° F.

5. *Polarised light examination.*—The solutions were examined with the polarising microscope. A few drops were placed on a microscope slide and covered with a glass square. The solutions were allowed to evaporate before examination. Control solutions made with excess of cholesterol in acetone and benzene were also examined.

	Approximate angle of extinction
(*a*) Acetone extract of turban tumours:	
Rhomboidal crystals, probably cholesterol . . .	90°
(b) Acetone shaken up with excess of cholesterol:	
Particulate birefringent material	40°
(*c*) Benzene extract of turban tumours:	
1. Particulate birefringent material	40°
2. A few rhomboidal crystals, probably cholesterol .	50°
(*d*) Benzene shaken up with excess of cholesterol:	
1. Particulate birefringent material	40°
2. A few rhomboidal crystals, probably cholesterol .	40°

Examination under the polarising microscope revealed the presence of cholesterol crystals in the acetone and benzene extracts. There was a difference in these crystals shown by the varying angles of extinction. The acetone extract was 90°, the benzene extract was 50° and the control solution of cholesterol dissolved in benzene was 40°. Cholesterol dissolved in acetone showed no cholesterol crystals but the birefringent material present had an angle of extinction of 40°.

Chemical examination of extracts.

The Liebermann-Burchard reaction for cholesterol confirmed that it was present in both extracts.

Experimental animals

One hundred and sixty mice of mixed colours, male and female, were obtained from the British Empire Cancer Campaign's farm at Chalfont St. Giles. They were between 2 and 3 months old and varied in weight from 20 to 41 g.

The sexes were segregated and housed in cages, 20 mice to each cage. They were fed on National Cattle Food 3, wheat and N.C.F. 3 with added cod liver oil was given once a week, and water was always freely available.

The fur on the backs of the mice from the occiput towards the tail for three-quarters of an inch was clipped short with scissors. In a few cases the skin of the backs of the mice was accidentally cut and these were excluded from the experimental series, leaving a total of 152. The clippings were repeated as the hair re-grew, but those mice painted with benzene showed little re-growth of hair whilst being painted.

The mice were given numbers from 1–152 and identified in their cages by combinations of triangular wedges removed from their ears with scissors. They were divided into four groups, of 22 males and 16 females, and painted with acetone extract, acetone, benzene extract and benzene respectively.

A perforated card index system was used and each numbered mouse had a card on which all details of experimental procedures, any abnormal findings, and post mortem findings, were entered.

Experimental painting

Number 4 Newton & Wright Indian sable hair paint brushes were used. Each brush was dipped into the appropriate fluid, and then the metal, which holds the hair, tapped gently against the neck of the bottle to shake off excess fluid. The solution was then applied to the clipped area of the neck with four strokes of the brush, twice a week for 10 months. The average weight of solution per painting was 0·472 mg.

During the last 3 months of this experiment 30 mice were lost due to infectious ectromelia and at the end of the experiment after deaths from all causes 87 of the original 152 remained.

SECOND EXPERIMENT

Exposure to ultra-violet light.—Twenty extract painted mice and 20 control animals from the previous experiment were then selected for irradiation with ultra-violet light.

Two metal cages were constructed each having 20 small compartments measuring 2 inches by 2½ inches by 1½ inches, and covered with a wire mesh lid. By confining the mice in these small compartments it was possible to keep the painted areas of the backs uppermost and so expose them to the ultra-violet radiation given from above the cages.

Ultra-violet radiation was given with a Hanovia Homesun lamp. This lamp was found to produce erythema of the human skin by 3 minutes' exposure at a distance of 3 feet.

The burner was placed 8½ inches from the backs of the mice. Irradiation was carried out twice weekly for 8 months, starting with 10 minutes for the first week, 15 minutes for the second week, and subsequently 20 minutes. This produced a severe erythema of the clipped areas and the ears.

Only 9 of the 40 mice used in this experiment survived the 8 months' irradiation. They were then about 2 years old.

RESULTS OF THE EXPERIMENTS

No tumour occurred during the whole course of the experiments in the experimental areas. Microscopical sections were prepared at intervals during both experiments from the interscapular regions and stained with haemotoxylin and eosin. In some cases a second section was stained with haemotoxylin and

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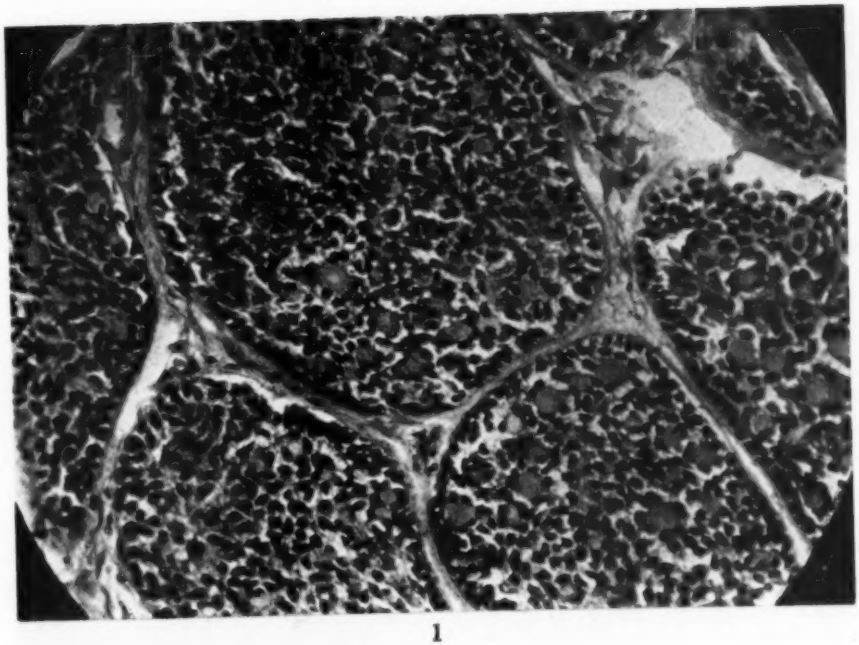
EXPLANATION OF PLATE

FIG. 1.—Shows limiting membrane and "cysts". × 400.



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tartrazine to demonstrate collagen and connective tissue. Certain changes, similar to those seen in areas of senile hyperkeratosis of the human skin, were found in sections from the extract painted animals, but not the controls, in the first experiment, and in all the animals exposed to large doses of ultra-violet light. The normal three or four layers of well formed cells of the epidermis were replaced by a thickened hyperplastic epidermis. These thickened areas were composed of many layers of cells which were larger, less well defined and poorly differentiated when compared with the normal epidermal cells. In the dermis there was chronic inflammatory reaction of granulation tissue infiltrated with inflammatory cells. These changes suggest that pre-cancerous dermatoses were produced by the extracts or ultra-violet light but frankly malignant changes were not seen in any of the sections.

SUMMARY

1. Acetone and benzene extracts of turban tumours were made. No spectroscopic evidence of known carcinogenic agents was found but fatty compounds obscured the regions in which the bands would be expected. The presence of cholesterol was proved by the microscope with polarized light and also chemically.

2. The extracts were painted on to the backs of mice twice weekly for 10 months and 40 mice from this experiment were then subjected to large doses of ultra-violet light twice weekly for 8 months.

3. Histological evidence of pre-cancerous dermatoses was present in sections from the extract painted animals but not the controls. Similar changes were found in all the mice exposed to ultra-violet light. No evidence of definite malignant change was found.

I am indebted to Dr. C. H. G. Price, Research Pathologist of the University of Bristol, who allowed me to keep the mice in the animal house and who gave me much helpful advice during these experiments. I wish to thank Mr. J. E. Hancock for the photomicrograph.

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THE EXPERIMENTAL IMPLANTATION OF TUMOUR CELLS IN THE URINARY TRACT

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ALTHOUGH the direct seeding of tumour cells on serous membranes and on meninges is believed to occur commonly, implantation of cancer upon the mucosa of hollow organs and on epidermis has been discounted by most pathologists and clinicians. It has been emphasized by Willis (1952) that most instances of so-called " epithelial implants " which appear in the medical literature can be explained on the basis of lymphatic or haematogenous spread, or of multiple primary growths. Nonetheless, very little experimental investigation has been carried out on the subject, in spite of its potential clinical importance. One notable exception has been the study of " aerial " spread of tumour cells in the respiratory tract. Several groups have investigated this problem, and all have reported that direct epithelial implantation does occur (Furth, 1946 ; Bronk and Appel, 1949 ; Norman and McBroom, 1957 ; Hershfield, 1957). These studies furnish convincing evidence that pulmonary tumours can spread directly throughout the bronchial tree.

In many other areas of human cancer the question seems to be an academic one : for example, even if implantation on intact gastrointestinal mucosa or on skin could be demonstrated experimentally, the evidence is strong that it does not represent an important clinical phenomenon. It might be noted that attempts at experimental implantation in these sites under various conditions have yielded negative results in our own hands.

For many years controversy has existed regarding the mode of spread of tumours of the urinary tract. The major question concerns the common finding of multiple tumours in the bladder and the frequent occurrence of what appear to be secondary tumours in the bladder or lower ureter from a renal pelvic tumour. Secondaries in the lower urinary tract from a primary kidney tumour seem to be much less common, although cases of this type have been reported (McAlpine, 1948 ; Hovenanian, 1950 ; Howell, 1951 ; Abeshouse, 1956), and have given rise to the same dispute as to their mode of spread. Four theories have evolved in the attempt to explain the occurrence of these multiple tumours : (1) Implantation or seeding in the urinary tract. (2) Lymphatic spread. (3) Direct extension. (4) Multicentric origin.

The " implantation theory " apparently was proposed first by Albarran and Imbert in 1903 and postulates that exfoliated tumour cells can implant directly on urothelium. It should be emphasized that this theory is contested by others mainly when applied to implantation on *intact* mucosa. The possibility of seeding upon raw surfaces or in surgical wounds is accepted far more widely, not merely in the urinary tract, but in many regions of the body. Examples of implantation

on traumatized urinary tract have been described by Kiefer (1953) and by Hinman (1956), and very little dispute has arisen over the validity of this claim. Implantation on intact mucosa remains a more contentious point. The proponents of this mechanism of spread have based most of their argument on the appearance and arrangement of the multiple tumours. There is a pronounced tendency for unilateral distribution in the pelvis and ureter, and for the occurrence of bladder secondaries near the ureteral orifice. MacAlpine (1947) and others have emphasized the finding of secondary tumours at points of narrowing of the urinary tract, such as the uretero-vesical junction and the bladder neck, and have suggested that cells may be held up at these points, thus favouring implantation.

Spread through submucosal lymphatics has been suggested as a principal method of spread by various writers from time to time, including Stevens (1920) and Saltzstein and Beaver (1940). Post mortem studies of advanced urinary tract tumours leaves little doubt that lymphatic spread does occur. However, McDonald and Priestley (1944), in their study, found relatively few cases of lymphatic involvement despite extensive spread. One objection to lymphatic spread as an explanation of the pathogenesis of multiple mucosal tumours is the fact that the normal lymphatic flow is up the ureter rather than down (Potampa and Calhoun, 1949), while most secondaries appear below the original tumour. It is possible that the flow might be reversed at times but it seems unsound to assume that such a mechanism is the principal method of spread in the absence of any real positive evidence.

Hunt (1929) suggested that multiple tumours represent continuous extension along the mucosa. Again, the onus would appear to rest upon the proponents of this theory to demonstrate histological continuity, and as far as we are aware, this has not been done.

Hansemann (1890) first suggested that the multiple urinary tumours represented not metastases, but independent primaries developing in tissue with a general neoplastic tendency. This theory has had a number of vigorous proponents who believe that it is unlikely that exfoliated cells could remain viable and implant on intact urinary epithelium, especially so long after the removal of the primary tumour (Ewing, 1928; Kaplan, MacDonald and Thompson, 1951; Baker and Graf, 1953). Mellicow (1952) has described microscopic lesions on grossly normal mucosa on bladders removed for papillary carcinoma; these included squamous metaplasia, hyperplasia and leukoplakia. These lesions were not found by him in normal bladders, and suggest that multiple atypical foci, which might form independent tumours, commonly exist in the presence of urinary tract tumours. Willis (1952) has argued that multiple primary tumours should be haphazard in distribution, and particularly that the unilateral action of urinary carcinogen is an untenable idea. The experimental work of McDonald and Lund (1954) suggests that urine may directly distribute carcinogens on the mucosa. If this is applicable to human urinary cancer, it would readily explain multiple tumour formation, and the character of the main urinary flow might produce a distribution of tumour that is not haphazard.

It is evident from the foregoing review that a long standing lack of agreement exists regarding the spread of urinary tract tumours. The conflicting views and reports perhaps emphasize the limitations of morphologic studies in establishing the pathogenesis of some lesions. Experimental studies, on the other hand, are fraught with their own peculiar pitfalls, and direct application to human disease

is constantly a matter of doubt. Nonetheless, such studies have proven of value in many fields, and attempts to solve the present problem by experimental studies have been surprisingly meagre. A notable exception has been the work of McDonald and Thorson (1956). These workers demonstrated tumour implantation in artificially constructed bladder pouches of four of seven dogs. These studies were remarkable in that transplantation of tumours between dogs was accomplished ; most such trials have had poor success with the exception of thyroid carcinoma in inbred or irradiated puppies (Allam *et al.*, 1954). Our own studies suggest that an absolutely intact mucosa is difficult to assure in an operated area, and that implantation occurs very readily on small defects.

In the present studies, a urinary tract tumour was not employed ; instead, the very anaplastic Walker 256 rat tumour was utilized. Use of this tumour is open to the valid criticism that it bears little resemblance to the comparatively well differentiated papillary carcinoma commonly encountered in the human urinary tract. It seemed reasonable to us, however, to first attempt to settle the basic question : can *any* tumour survive and implant in the urinary tract ? The results we have obtained may suggest the value of further trials using a typical tumour of transitional epithelium. A preliminary report of this work has been

EXPLANATION OF PLATES

All sections were stained with haematoxylin and eosin.

FIG. 1.—Walker 256 tumour growing in bladder mucosa 7 days after injection of tumour suspension by catheter. The bladder received no trauma. Note the superficial position of the tumour. × 11.

FIG. 2.—Walker 256 tumour 9 days after inoculation by catheter into an intact bladder. Note invasion of muscularis. × 15.

FIG. 3.—Bladder tumour 5 days after inoculation. Minute bladder lesion, not grossly visible, and covered at all levels examined by an intact layer of epithelium. × 52.

FIG. 4 and 5.—Sections of bladder taken serially 24 hours after injection of Walker 256 tumour. Single large atypical cells are seen in the epithelium, two of which are in mitosis. These probably represent Walker 256 tumour cells. × 480.

FIG. 6.—From serial sections of bladder taken 1 hour after injection of Walker 256 tumour by catheter. A defect is present in the mucosa and tumour cells have implanted in the fibrinous exudate. Care was taken to avoid trauma to this bladder. × 210.

FIG. 7.—Normal rat bladder in contracted state showing transitional epithelium many cells in thickness. × 52.

FIG. 8.—Normal rat bladder in distended state showing single layer of low cuboidal cells forming the covering epithelium. × 122.

FIG. 9.—Normal rat kidney showing (above) the cortex, and (below) the papilla, with deep recesses between pyramid and cortex. × 13.

FIG. 10.—Typical tumour nodules, seen on either side of the right renal pyramid 19 days after injection of Walker 256 tumour into the left ureter. × 12.

FIG. 11.—Section showing renal papilla (left) and renal pelvis (right). High columnar epithelium, mainly a single layer, covers the papilla. The pelvic epithelium consists of a typical thick transitional layer. × 100.

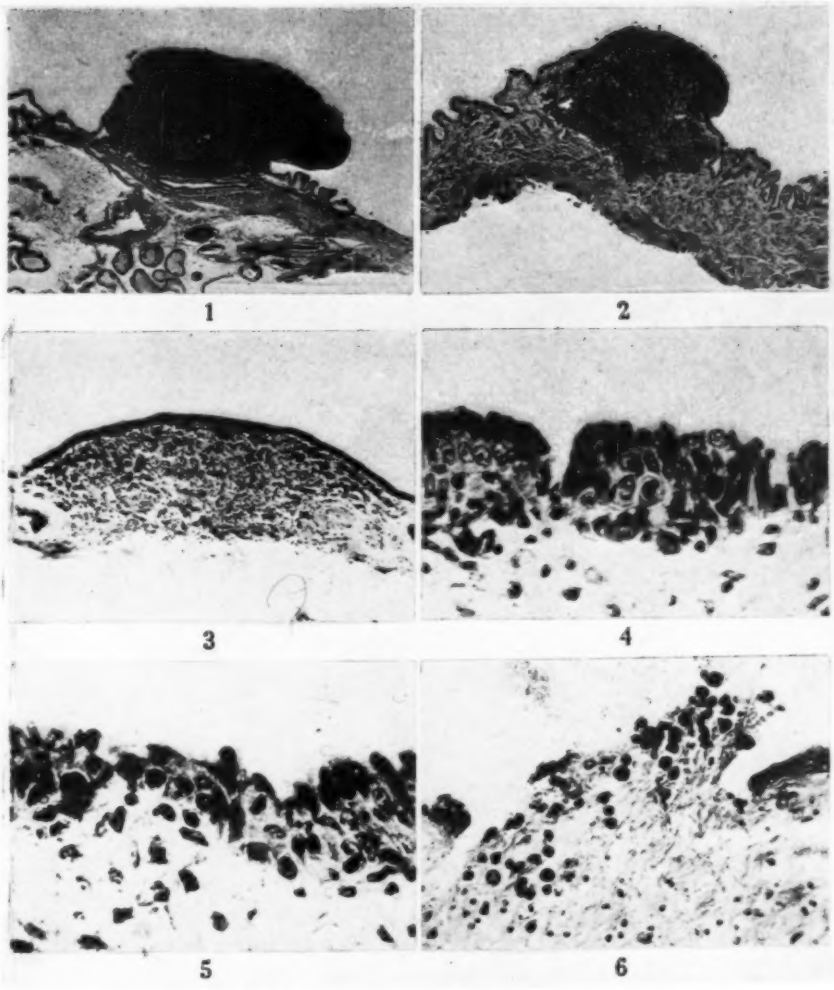
FIG. 12.—Section of a deep recess between cortex and pyramid. These clefts in the rat are lined by a single, very flat layer of cells reminiscent of peritoneum. × 300.

FIG. 13.—Section of kidney pyramid 5 hours after injection of Walker 256 tumour into the bladder by catheter. The normal covering layer is replaced by tumour cells, one in mitosis. × 530.

FIG. 14.—Section from the same kidney as Fig. 13 showing tumour cells covering both sides of the cleft between cortex and pyramid. The cells remain superficial at this stage, and appear to grow among the normal lining cells. × 500.

FIG. 15.—Massive replacement of normal epithelium in recess between cortex and pyramid 6 hours after introduction of tumour cells into the bladder. × 265.

FIG. 16.—Tumour cells lying free in cleft of kidney 5 hours after injection into bladder by catheter. × 265.



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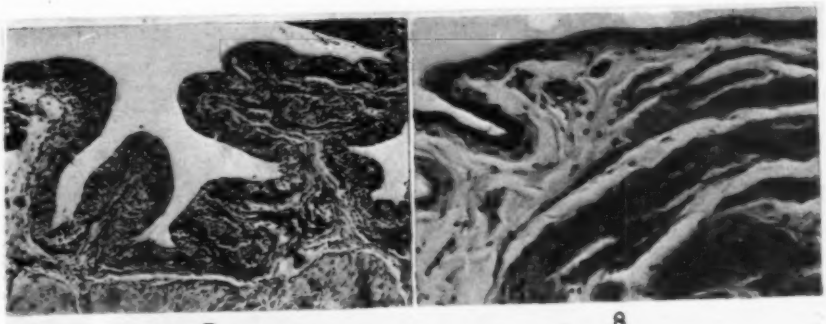

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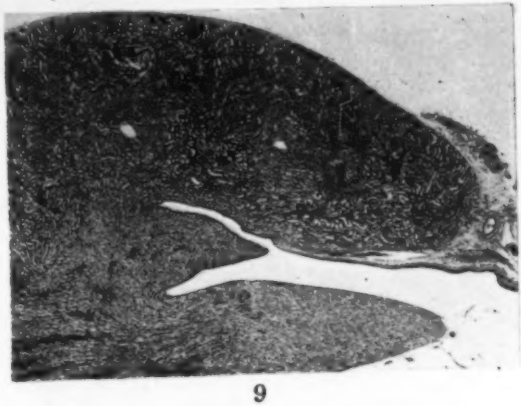

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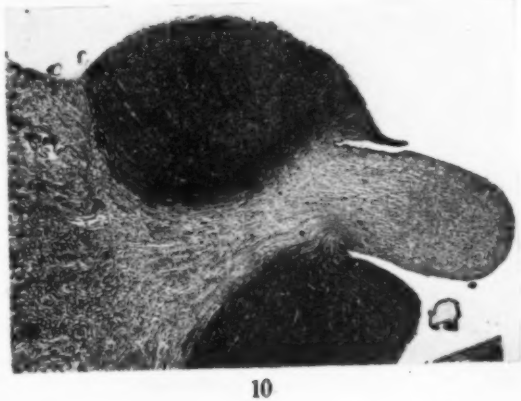

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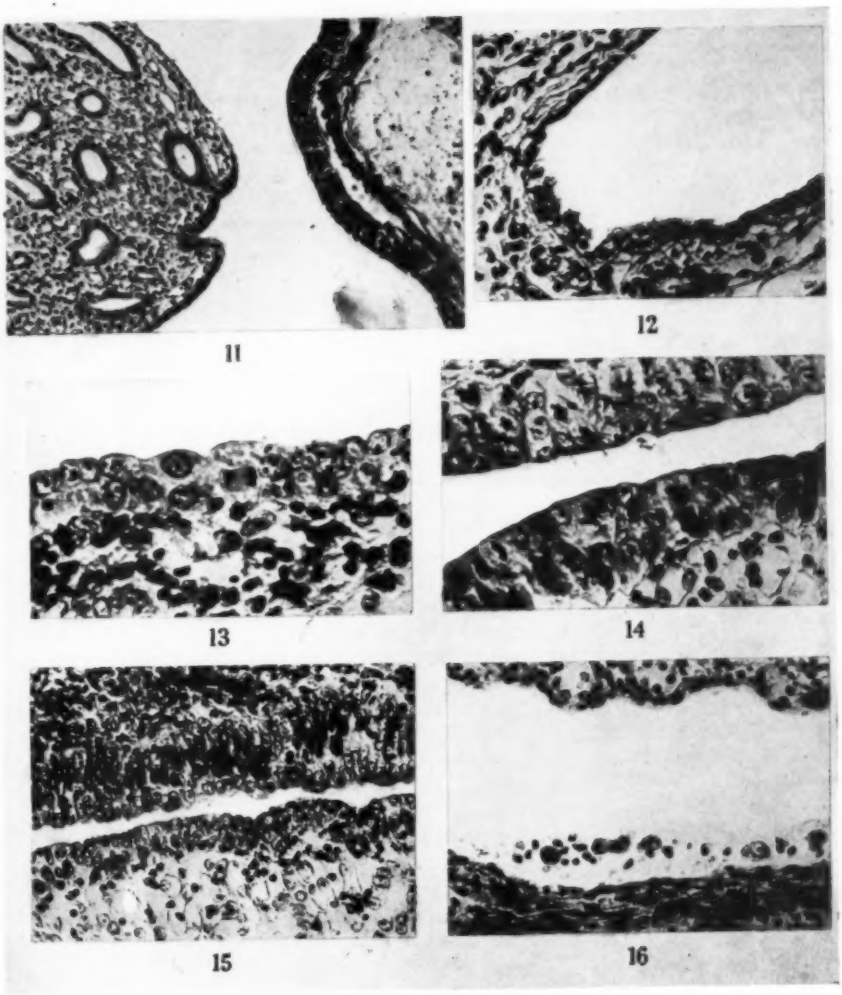

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previously given (Wallace and Hershfield, 1957); the present paper reports an extended study with further trials and more detailed histological studies.

MATERIALS AND METHODS

Walker 256 carcino-sarcoma was used throughout the experiments, and all transplantations were performed on young mature male Sprague-Dawley rats. Stock tumour was removed under aseptic conditions and chopped in saline with scalpel blades. The minced tumour was then further dispersed by passage through a 10 c.c. syringe. The suspension was allowed to settle, and the supernatant fluid, containing small clumps and single cells, was used for injection. Trypan blue (0·1 per cent) was added to furnish an easily visualized dye which helped to indicate where the injections had gone in some of the experiments.

A. Ureteral injections

Twenty-two rats received an injection of tumour cells into the left ureter. Rats were lightly anaesthetized with nembutal, and the anaesthesia was made complete with ether. The skin over the left loin was shaved and washed with 80 per cent alcohol. An incision was made in the left lumbar area, exposing the perinephric fat. The kidney was delivered into the operative wound, and the renal vessels were ligated and severed. The ureter was left attached, but was ligated adjacent to the kidney pelvis. Two loops of suture were placed around the ureter, one and one-fourth inches from the kidney but were not drawn tight. A small incision was then cut in the ureter with fine scissors, and a 27-gauge needle with a blunt end was inserted, pointing downward. The sutures were then drawn tightly around the needle. A tuberculin syringe containing tumour suspension was then attached, and the injection was made down the ureter into the bladder. The volume injected in this experiment was approximately 0·5 c.c. Immediately after injection the bladders of 11 of the rats were traumatized. This was accomplished by pinching the dome from the peritoneal surface momentarily with a haemostat. The other 11 rats received no bladder trauma. The needle was withdrawn, tying the ureter at two levels during the withdrawal. The ureter was severed just above the incision, and the kidney with the proximal ureteral stump was removed. The operative area, including the distal ureteral stump, was swabbed with 80 per cent alcohol and then saline in an attempt to destroy any tumour cells which might have been spilled in the operative area. The muscle and skin were closed with black silk sutures.

B. Urethral injections

The occurrence of local tumour at the ureteral stump in the above experiments indicated the need for an alternative route of administration. It was found that injection of fluid by catheter into the bladder via the urethra was feasible, initial trials made with the bladder exposed revealing the presence of injected dye immediately in the bladder. A number 10 polyethylene catheter was attached to a 23-gauge needle and was inserted into the penile urethra for approximately one centimeter. The catheter was never introduced all the way into the bladder, and in initial trials some difficulty was experienced with reflux around the catheter. It was found that, if firm digital pressure was maintained around the penis during injection, the fluid readily entered the bladder and there was little or no reflux.

The first experiment using catheter injections utilized 24 rats. Each received 1 c.c. of a dilute tumour suspension, and 5 rats received trauma to the bladder as previously described. The other 19 had sham operations but no bladder trauma. Animals died at 8–16 days, or were killed at 16 days. Rats in this experiment suffered from severe cystitis, pyelonephritis and prostatitis, and for this reason penicillin and streptomycin were added to the tumour suspensions in subsequent experiments.

A further 26 animals were injected with a slightly higher volume of tumour suspension (1·5 c.c.) and a heavier concentration of cells was used. All injections were done as before by catheter, and none of the rats received bladder trauma. In an effort to locate earlier lesions in bladder and kidneys, the animals were killed at earlier stages (3 days, 5 days and 7 days).

An additional group of 30 rats was given a similar injection of cells by catheter and the animals were killed at very short time intervals (1 hour, 6 hours, 24 hours and 48 hours). Kidneys and bladders of some of these animals were subjected to serial sectioning in a further effort to locate early foci of implantation. The studies on this group are incomplete in that not all the material has been studied completely because of the amount of histological work involved.

C. Urethral injections with ureter blocked

As an additional study of the pathogenesis of tumours appearing in the kidney (*vide infra*), the effect of blocking one ureter was studied. It was felt that ligation was not a suitable method of blockage, since venous and lymphatic channels would also be interrupted, and these represented other possible routes of tumour spread to the kidney. For this reason the lumen was internally blocked by the injection of agar. A tiny incision was made in the left ureter and a 27-gauge needle inserted upwards toward the renal pelvis. Approximately 0·2 c.c. of melted agar at a temperature of 45° C. was injected. The agar rapidly solidified, blocking the upper ureter. The incision in the ureter, however, was left open after the needle was removed. Three days later Walker 256 tumour suspension was injected by urethra, and 9 days later the animals were killed and their kidneys examined for tumours.

RESULTS

A. Ureteral injections

The results of this study are summarized in Table I. Rats having normal bladders showed no bladder tumours at the termination of the experiment. Eight of the 11 rats having traumatized bladders presented with huge tumours which nearly filled the bladder lumen in some animals. An unexpected finding was the presence of tumours in the right kidney in 5 of the animals, 4 of which

TABLE I.—*Effect of Injection of Walker 256 Tumour into the Left Ureter*

Group	Number of rats	Duration of experiment (days)	Bladder tumours	Kidney tumours	Other tumours
Intact bladder	11	18–20	0	1	Ureteral stump and peritoneum.
Traumatized bladder	11	21	8	4	Ureteral stump and peritoneum.

had traumatized bladders. A detailed description of the tumours is given below. The renal tumours in this experiment were large (5–10 mm.) and consisted of a single mass replacing either the entire renal pyramid or one side of the papilla.

B. Urethral injections

The results of the first study using urethral injections are given in Table II. Three of the 5 traumatized bladders showed tumours ; 1 of the remaining 2 was eaten before autopsy could be done. Of 19 rats having intact bladders, one displayed a tumour which did not differ from those in the traumatized group. Kidney tumours again appeared in 8 of the 19 normal rats, and in 1 of the 5 "traumatized" group. These were identical to those produced with ureteral injections, occurring on the sides of the renal pyramids. A single animal developed a tumour in the penile urethra, probably due to direct implantation associated with trauma. None of the animals showed tumour in peritoneum, lymph nodes or other sites. The experiment was complicated, however, by the occurrence of prostatitis, cystitis and pyelonephritis in some rats. The addition of antibiotics to the tumour suspension in subsequent experiments, supplemented by intramuscular antibiotics, completely overcame this difficulty.

TABLE II.—*Effect of Injection of Walker 256 Tumour into Urethra*

(First Experiment)

Group	Number of rats	Duration of experiment (days)	Bladder tumours	Kidney tumours	Other tumours
Intact bladder	19	13–16	1	3 unilateral 5 bilateral	1 in penile urethra.
Traumatized bladder	5*	8–11	3	1 unilateral	None.

* Bladder of one rat was lost.

In Table III are shown the results of catheter injection of a larger and more concentrated tumour suspension to a further 26 animals. None of this group received bladder trauma, and examinations were made at earlier intervals than in the first experiment. A single small bladder tumour was found microscopically at 5 days ; while at 7 days, 7 of 16 animals displayed grossly visible bladder tumours. One renal tumour was present at 3 days, and of the 26 rats injected a total of 14 developed kidney tumours, 8 unilateral and 6 bilateral.

A complete report cannot be given on the additional group of 30 rats similarly injected by catheter and killed at very short intervals (1–48 hours). Serial sections were made on a portion of these in a further effort to locate early tumours ; these are described below.

TABLE III.—*Effect of Injection of Walker 256 Tumour into Urethra*

(Second Experiment)

Duration of experiment (days)	Number of rats	Bladder tumours	Kidney tumours	Other tumours
3	5	0	1 unilateral	None.
5	5	1	4 unilateral	,,
7	16	7	3 unilateral 6 bilateral	,,

C. Urethral injections with ureter blocked

Ten rats survived the duration of the experiment and were autopsied 9 days after the urethral injections (12 days after the left ureter was blocked). Two displayed bladder tumours. Five of the animals had tumours in the right kidney, while none had tumours in the left kidney. The likelihood of this distribution being due to chance is less than 0·01 (Mainland, 1948). Seven of the 10 animals had tumour in the retroperitoneal tissues around the incised left ureter. We attribute the presence of tumour here to the escape of tumour cells out of the ureter through the defect left in the ureteral wall.

Morphological Description of the Tumours Produced

(a) *Bladder tumours.*—The bladder tumours had the same gross and microscopic appearance regardless of whether they followed ureteral or urethral injections, and whether they occurred on intact or traumatized epithelium. They appeared to originate on or in bladder mucosa, and even at a late stage were often still confined to the mucosa and submucosa (Fig. 1). Invasion of the wall did occur (Fig. 2) but in no case was the peritoneum involved. The larger tumours at 14–21 days completely filled the bladder and were sometimes multiple. Hydronephrosis accompanied a few of these late lesions. Although the smaller lesions were very superficial, a few were covered completely by epithelium (Fig. 3) and suggest that implantation may be followed by overgrowth by epithelial cells.

A search by serial sections for the earliest stages of implantation on intact bladder proved an arduous task, but a few foci were identified. These consisted of small groups of tumour cells among the epithelial cells, many in mitosis. They presented as many single cells rather than discrete nodules, and were difficult to positively identify, except by their size, atypical appearance, and mitoses (Fig. 4 and 5).

It must be noted also that, in at least 3 animals, minute areas of ulceration were found in non-traumatized bladders, and tumour was present in 2 of these areas (Fig. 6). This raises the question of whether *all* instances of implantation even on allegedly normal mucosa actually took place in small defects. While it is virtually impossible to exclude an extremely minute defect occurring *previously*, serial sections of some bladders showed definite implantation with no evidence of any ulceration whatsoever. If mucosal defects did occur in these cases they were so minute that for practical purposes the bladders must be considered intact. Aside from the presence of actual defects, the thickness of the bladder mucosa varied tremendously, from a thick pseudostratified layer in a contracted bladder to a thin, single layer of cuboidal cells in a dilated bladder (Fig. 7 and 8). Possibly these variations may have also played a part in the success or failure of implantation.

(b) *Kidney tumours.*—The renal tumours were an unexpected finding, and occurred commonly in all groups of animals. Their appearance and location was likewise very constant. In a moderately advanced stage they consisted of spherical masses which obliterated the recesses between cortex and medulla (Fig. 9 and 10). Their exact initial site was determined by studies of earlier lesions, with serial sections to locate the earliest foci. Early tumours never occurred within the renal parenchyma, nor did they appear on pelvic epithelium or on the central portion of the papillae. These surfaces, in the rat, are covered with high columnar and

pseudostratified epithelium (Fig. 11). The deep recesses between cortex and pyramid, however, are normally lined by only a very thin flattened layer of cells more reminiscent of peritoneum than of urinary epithelium (Fig. 12). It was in these recesses that early lesions were always located, and serial sections of the earliest studies showed tumour cells in and immediately beneath the epithelium with formation of flat areas of replacement by tumour (Fig. 13, 14 and 15). Tumour cells were also found lying free within the recesses (Fig. 16). No evidence of pre-existing ulceration or inflammation was seen, and it seems almost certain that implantation occurred on intact epithelium. It may be suggested that the very thin lining in the recesses may be more susceptible to implantation than other areas, and resembles peritoneum in its susceptibility. Also, the deep recesses may represent a more "sheltered" region away from the main urinary flow, where cells are better able to survive.

DISCUSSION

The initial studies, performed with the use of a fairly dilute tumour suspension and a relatively small inoculum, established clearly that traumatized epithelium is susceptible to implantation, but suggested that intact epithelium was resistant. Use of heavier suspensions and larger volumes produced a far higher incidence of implantation on normal bladder mucosa. We believe that implantation upon normal bladder mucosa has been demonstrated; or at least on mucosa which was essentially normal and had not been traumatized. If any defect is required for implantation to occur, it must be extremely minute in some cases, certainly no more than might be found in many human bladders in the presence of obstruction. It is difficult, also, to imagine any route except the urinary passages by which the tumour cells could have reached the bladder mucosa in the present experiments.

There is likewise no evidence that the renal tumours implanted exclusively on traumatized areas, since they occurred in crevices well sheltered from any external influence, and no ulceration or inflammation was seen in conjunction with the early tumours. The appearance and location of the early tumours, and the presence of tumour cells lying loose in the crevices immediately after injection by catheter, indicate that the tumour cells reached the kidneys by retrograde spread up the ureters. In other experiments we have found that trypan blue injected into the bladder in as little a volume as 0·25 c.c. can be seen to enter the ureters immediately. This reflux is not a surprising phenomenon; it has been observed with some human beings, especially in children, even with normal ureters.

It should be re-emphasized that the tumour used in the present experiment was not of urinary tract origin, and was far more malignant than the typical human tumour of renal pelvis, ureter or bladder. Also, the inoculum given was very large in the number of cells, probably much greater than would ever occur from exfoliation from a human tumour. On the other hand, only a single exposure occurred in the experiments, while human tumours may exfoliate into the lumen for years. It must also be borne in mind that implantation on minute defects occurred very readily; such defects might commonly exist in *apparently* normal human urinary tract, especially when infection or obstruction are present. These conditions often do exist when urinary tract tumours are present.

The present studies do not give a direct answer to the question of whether

human urinary tract tumours spread by the urinary stream. They do indicate, however, *first*, that some tumour cells will survive in the urine long enough to implant successfully; *second*, that they will implant very readily on very small mucosal defects, and under some circumstances, on intact epithelium.

SUMMARY

The ability of Walker 256 tumour to implant upon rat urinary mucosa has been studied by injections of cells into the ureter and urethra. Implantation occurred on a high proportion of bladders which had been traumatized, and upon some apparently intact bladders. Tumour cells also implanted in the kidneys, apparently secondary to retrograde passage up the ureters. The significance of these findings has been discussed.

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THREE NEW VIRUS-INDUCED FOWL SARCOMATA

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SPONTANEOUS sarcomas of the domestic fowl are relatively common (Olson and Bullis, 1942; Campbell, 1945) while leukaemias not infrequently reach epizootic levels in commercial flocks (Olson, 1948). Both of these are considered to be of viral origin although this point is very rarely investigated. In consequence, the characterisation of the viruses is hardly ever attempted. Their viral origin therefore remains in most instances an unproven assumption. Hence any attempt to consider in a scientific manner the epidemiology of either condition founders at once in the absence of information concerning the number and mutability of any viruses present in a particular flock.

The present account deals with three new sarcomata isolated at this Centre and arising from spontaneous cases submitted to the Ministry of Agriculture's diagnostic laboratory at Lasswade. The co-operation of their staff is appreciated.

Isolation

These tumours were selected as being reasonably fresh and uncontaminated. Only mucoid tumours were considered. While at least one non-mucoid virus-associated tumour of the fowl is known (the MH_2 reticuloendothelioma of Murray and Begg, 1930), restriction to mucinous tumours simplified the problems of preliminary selection. That all three are myxomatous fibrosarcomata is therefore not significant.

A portion of the material as received was ground with 5–6 per cent glucose to a fine suspension, penicillin added, and injected intramuscularly into young chicks. The number and age depended upon what was available at the time, and did not seem to be significant. From the resulting growths the material for further study was obtained. In each case the result of the passage resembled the original tumours, so that it is reasonable to assume that the virus was the cause of these, and was not a contaminant passenger isolated from a lesion it had not induced.

Morphology

P.R.C. II. This arose in the mesentery of a Leghorn cross-bred. The primary was a rather vascular tumour composed of interlacing bands of loosely-arranged spindle cells often separated by much acidophilic structureless material. This stained positively with Mayer's mucicarmine and especially vigorously with periodic acid Schiff stain. Some rounded cells were present, especially in the acidophile zone where they tended to lie within a clear space. Nuclei were fairly

constant in size, containing finely aggregated chromatin and an occasional prominent nucleolus.

The first passage produced interlacing bands of rather plumper cells arranged more compactly and with less intercellular material. Mitoses were numerous and the nuclei were hypertrophied and with prominent, sometimes multiple, nucleoli. At the edge of the tumour, which showed a peripheral infiltration of lymphocytes, the cells tended to lose their spindle shape as they invaded the muscles, but retained definite cell boundaries, whereas the cells constituting the body of the tumour had a syncitial appearance. Occasional single or double cells were found in clear spaces in the mucinous matrix.

On further passage there was some increase in pleomorphism, but the general appearance remained unchanged. Regressions were scarcely ever encountered. Metastases to the liver and lungs were found on occasion and sometimes the "haemorrhagic disease" of Duran-Reynals (1940*b*) was noted in young chicks.

P.R.C. III. This originally occurred in the muscles of a Rhode Island Red female. There was much necrobiosis. The cells were somewhat more pleomorphic than in Tumour II, tending to be plumper and exhibiting cytoplasmic vacuolations.

Subsequent passages can be briefly summarised by stating that the tumour closely resembled the Rous I sarcoma. Lung metastases were often present, regressions were rather frequent, and in young chicks haemorrhagic lesions were sometimes noted.

P.R.C. IV was found in the ovary and mesentery of a Light Sussex female, the latter site most probably representing the primary growth. It was composed of slender, loosely interlacing spindle cells with dense nuclei, with substantial intercellular spaces occupied by a bluish matrix with a fine eosinophilic fibrillar structure.

On passage, a rather pleomorphic tumour was obtained. Some cells resembled mature fibroblasts, though mitoses were frequent, and rounded forms resembling histiocytes were also present. Giant nuclei were sometimes noted. There was a plentiful but rather weakly staining mucinous matrix. Lymphocytic and granulocytic infiltration occurred. Metastases were rare, and almost entirely confined to the liver or spleen. A special feature, prominent in the splenic metastases, was the presence of beaded clefts running in all directions. Haemorrhagic disease was never seen when young chicks were used for passage.

Virus

All three tumours could be transmitted by cell-free extracts, and in the case of III and IV birds whose tumours regressed contained antibodies in their blood which completely neutralised the tumour-producing activity of such extracts. It only remains to add that in this work nothing was encountered to suggest that these viruses differ fundamentally from the other fowl sarcoma viruses.

Serological Methods

Antisera.—These were obtained from birds bearing small tumours or those in which a tumour had regressed. Blood was taken at least 50 days after inoculation by which time the bird was expected to be a fully-immune carrier (Carr, 1944). Serum was heated to 53° for 2 hours, and then kept in the refrigerator until needed. The same batch of antiserum was used for all tests.

Virus.—This was a suspension purified according to the method of Bather (1953) in the case of the Rous I tumour, and a cell-free tumour extract, suitably diluted, for the other tumours. Its infectivity was titrated at the same time in young chicks according to the method of Carr and Harris (1951) with the corrections of Parker and Rivers (1936).

Neutralisation.—The standard method was used. Equal volumes of a stock dilution of virus, and a dilution of the antiserum were left for at least one hour to react, then 0·4 ml. of the mixture was injected into the breast or leg muscles of a group of 4–5 chicks, while a control of virus + saline was inoculated into the opposite site. The size of the resulting tumours is proportional to the amount of unneutralised virus.

At the end of 28 days, surviving birds were killed and the results recorded.

A typical result, showing partial neutralisation of III virus with Rous antiserum is shown in Table I.

The full neutralisation results are summarised in Table II.

No effective antiserum against II was obtained, as all inoculated birds grew a progressive tumour which killed them before 50 days had elapsed.

It is clear from this that III and Rous are closely related, but not identical. Since III antiserum completely neutralises Rous I, while the reverse neutralisation

TABLE I.—*Neutralisation of III Virus by Rous I Antiserum*

Site	Inoculum	Tumour size Bird No. 1	2	3	4
R. breast	III virus + Rous antiserum	−	−	−	−
L. breast	„ + saline	++	++	++++	++++
R. leg	„ + Rous antiserum × 1/5	±	±	+	+
L. leg	„ + saline	++	++	++++	++++
		5	6	7	8
R. breast	„ + Rous antiserum × 1/25	+++	+++	+++	+++
L. breast	„ + saline	++++	++++	++++	++++

Tumour size is indicated by the number of + signs.

TABLE II.—*Degree of Neutralisation of Viruses by Antisera*

Virus	Infectivity	Antiserum	Final dilution 1/2	1/10	1/50
Rous	10^{3}	Rous	C	C	C
II	$10^{4.3}$	„	0	0	0
III	$10^{3.3}$	„	C	p	t
IV	$10^{3.3}$	„	0	0	0
			1/2	1/8	1/32	1/128
Rous	$10^{3.3}$	III	C	C	C	C
II	10^{3}	„	p	t	0	0
III	$10^{3.5}$	„	C	C	C	C
IV	$10^{3.3}$	„	p	t	0	0
Rous	$10^{3.3}$	IV	0	0	0	0
II	10^{3}	„	0	0	0	0
III	$10^{3.5}$	„	0	0	0	0
IV	$10^{3.3}$	„	C	C	C	C

Decreasing degrees of neutralisation are indicated as follows: C = complete, p = partial, t = trace, 0 = none.

is partial, it may be that III contains an antigen additional to those present in Rous I. The remaining viruses II and IV are distinct from both these and also from each other. The minor relationship of Rous I and II and III and IV, are of negligible importance, and there is always a doubt whether such weak reactions may not be due to naturally-acquired antibodies (Amies, 1937 ; Duran-Reynals, 1940*a*). This could only be decided, should the information be considered of importance, by repeated experiments using several antisera.

DISCUSSION

These three tumours, derived from the first three specimens investigated, have yielded three quite distinct viruses. Such tumours are by no means rare, and the number of types of virus may therefore be great. One of these viruses has been identified as belonging to the Rous I group, the others are at present unclassified. That this cannot be done is mainly owing to the dearth of information in the literature. The last serious attempt at serological classification of the fowl neoplastic viruses was that of Andrewes in 1933. To-day, so far as is known, there is no place with a collection of known tumour viruses, let alone of antisera.

In view of the importance of classification as a preliminary to study, this position seems extraordinary. At present, it cannot even be decided whether the sarcoma viruses form many groups (the Rous I group and MH_2–RF_4 group of Andrewes (1933), etc.) or consist of a single group of immutable variants deriving from a single virus type, or are mutated varieties of either arising in the field. For this reason also their relationship (if any) with the erythroleukoses and lymphomatoses remain hypothetical, though by all other criteria they are obviously very similar to the former.

In the present series, the relationship of III and Rous I was suggested histologically from the beginning, and the serological confirmation of this was not unexpected. If histological criteria could be used, even in part, as a substitute for the laborious serological classification detailed here, this would be a notable preliminary simplification of the problems of distribution of viruses met in the field. In any case, this draws attention to the need for the development of a simpler method for making such identifications. The method used here, though sensitive and delicate as a research technique, is unsuitable for routine diagnostic work, even if classification could reduce the number of cross-tests needed to a few standard antisera. Unfortunately, the simple but precise methods based upon haemagglutination are not applicable to these or any other tumour viruses (Carr, unpublished).

No variation in any of these tumours has so far been noted, though this has sometimes been a marked feature of virus tumours after isolation, e.g. the reversion of the osteochondrosarcoma (Rous, Murphy and Tytler, 1912) to an undifferentiated type and the changes of Rous I itself (Rous, 1910). To what extent this is correlated with changes in the antigenic structure of the virus is unknown ; biochemical change there must be, of course. Such instability seems to be rather frequent, and indicates that care must be taken to preserve the original strain of virus as isolated.

SUMMARY

Three new neoplastic visuses have been isolated from spontaneous myxomatous fibrosarcomata of the domestic fowl. One is closely related to Rous I serologically,

though not identical with it, and the sarcomata it induced are similar in structure and behaviour. The other two are unrelated to this and each other.

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DISTRIBUTION STUDIES WITH SULFUR 35-LABELED DISULFONAMIDES IN TUMOR-BEARING AND TUMOR-FREE MICE

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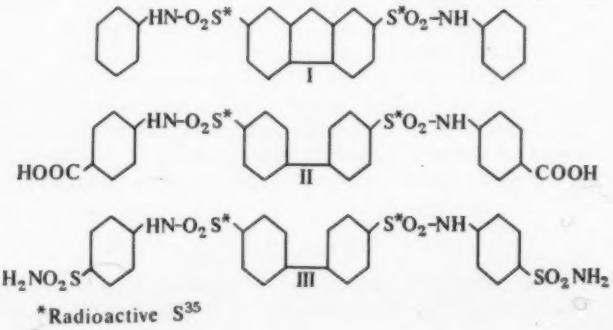
THE desirability of finding a radioactive compound that would localize in tumor tissue and which could be used in the diagnosis and therapy of internal cancer has long been recognized. Previous studies carried out in our Laboratory have shown that with certain sulfur 35-labeled derivatives of fluorene, localization in tumor tissue is favorable compared to liver, kidneys, spleen, blood and muscle. The first of these promising compounds was disodium fluorene-2,7-disulfonate-S^{35} (Argus, 1953). This compound was then used as the basis for the synthesis of a number of related substances which could be used for investigating the relation of chemical structure to tumor localizing properties in the hope of obtaining compounds of improved characteristics. Of those tested, sulfonated fluorene-2,7-di-(sulfonamido-2′-naphthalene)-S^{35} showed the best tumor localization. Two compounds without free sulfonic acid groups, fluorene-2,7-disulfonamide-S^{35} and fluorene-2,7-di-(sulfonamido-2′-naphthalene)-S^{35}, did not selectively concentrate in tumor tissue (Argus and Hewson, 1954). There seemed to be some indication at 8 hours, however, that the latter compound showed a differential in the blood between tumor-bearers and non-tumor-bearers. This difference was not large but if real could be of considerable diagnostic importance since there was the possibility that the effect could be enhanced in a new compound of suitable structure.

In the present study three new substances were investigated in tumor-bearing and tumor-free mice. These were fluorene-2,7-di-(sulfonamidobenzene)-S^{35} (I), a disulfonamido derivative of fluorene with molecular weight falling between the compounds previously tested ; and two related biphenyl derivatives, biphenyl-4, 4′-di-(sulfonamidobenzene-4-carboxylic acid)-S^{35} (II) and biphenyl-4,4′-di-(sulfonamidobenzene-S^{35}-4-sulfonamide) (III).



*Radioactive S^{35}

MATERIALS AND METHODS

Fluorene-2,7-disulfonyl chloride-S^{35}.—The procedure described previously (Argus and Hewson, 1954) was employed, but 40 millicuries of sulfur-35 were used with the same quantities of other reactants. Recrystallization from toluene gave a product melting 221–222°; specific activity was not determined on this intermediate.

Fluorene-2,7-di-(sulfonamidobenzene)-S^{35} (I).—Finely powdered fluorene-2,7-disulfonyl chloride-S^{35}, 1·82 g. (0·005 moles), was slowly added to a solution of freshly distilled aniline, 2·47 ml. (0·027 moles), in 35 ml. anhydrous benzene at 45°. This mixture was stirred for 2 hours at 60–70° and for 1 hour additional. The white precipitate was collected and washed free of aniline hydrochloride with hot water. Two grams of product (83·7 per cent of theory) melting at 250–255° were obtained. Following recrystallization from 95 per cent ethanol, the compound melted at 262–263° and had a specific activity of 17,000 disintegrations per second per mg. Analyses gave 13·35 and 13·59 per cent S, 62·91 and 63·27 per cent C, 4·02 and 4·16 per cent H, and 5·54 and 5·47 per cent N; the calculated values are 13·46 per cent S, 63·00 per cent C, 4·23 per cent H, and 5·88 per cent N.

Biphenyl-4,4'-disulfonyl chloride-S^{35}.—Biphenyl, 7·7 g. (0·05 moles), was added slowly with stirring to 16·3 ml. (0·25 moles) chlorosulfonic acid containing 20 millicuries sulfur-35. After the reaction mixture was agitated at room temperature for 4 hours, the light gray precipitate was collected, washed free of acid with cold water, and dried over phosphorus pentoxide, Two recrystallizations from anhydrous benzene gave 5·3 g. (30·22 per cent theory) of white product melting 206–208°. Analyses gave 19·57 per cent Cl, and 18·25 and 18·34 per cent S; the calculated values are 20·19 per cent Cl, and 18·26 per cent S.

Biphenyl-4,4'-di-(sulfonamidobenzene-4-carboxylic acid)-S^{35} (II).—Biphenyl-4,4'-disulfonyl chloride-S^{35}, 1·7 g. (0·005 moles), was finely powdered and slowly added to a solution of *p*-aminobenzoic acid, 2·8 g. (0·020 moles), in 30 ml. anhydrous acetone. The mixture was stirred for 30 minutes at reflux temperature and for 10 hours at room temperature. The light gray precipitate was collected, washed with 750 ml. hot water and dried over phosphorus pentoxide. The yield was 2·6 g. (82·6 per cent of theory); the compound melted at 320°. Recrystallization by dissolving in 70 ml. of a 2 : 5 mixture of water : dimethylformamide and precipitating with 30 ml. of water gave a white product melting at 327° and having a specific activity of 16,000 disintegrations per second per mg. Analyses gave 56·15 and 56·20 per cent C, 4·96 and 5·03 per cent N, and 11·26 and 11·28 per cent S; the calculated values are 56·51 per cent C, 5·07 per cent N, and 11·61 per cent S.

Biphenyl-4,4'-di-(sulfonamidobenzene-S^{35}-4-sulfonamide) (III).—Biphenyl-4,4'-disulfonyl chloride-S^{35}, 3 g. (0·0085 moles), dissolved in 35 ml. of anhydrous acetone was added with stirring to a solution of sulfanilamide, 7·35 g. (0·42 moles), in 25 ml. anhydrous acetone at 50°. The reaction mixture was stirred 1·5 hours at 45–50° and 10 hours at room temperature. A white precipitate was formed by slowly pouring the reaction solution into 400 ml. of water, with stirring. The product was collected, washed with 750 ml. of hot water, and air dried. This gave 5·2 g. (97·77 per cent of theory) of the compound melting at 289–290°. Recrystallization by dissolving in 70 ml. of a 2 : 5 mixture of water : dimethylformamide and precipitating with 30 ml. of water resulted in a product melting at 295°

and having a specific activity of 9700 disintegrations per second per mg. Analyses gave 46·59 per cent C, 9·18 and 9·23 per cent N, and 20·93 per cent S; the calculated values are 46·29 per cent C, 9·00 per cent N, and 20·60 per cent S.

Animal experiments

Male CAF_1/Jax hybrid mice were employed in this study. The distribution of the three sulfur 35-labeled disulfonamides was studied in 70 mice (Tables I and III) when the animals were 8 weeks old. Four weeks prior to administration of the compounds, 34 of the mice received a subaxillary transplant of a keratinizing squamous cell carcinoma (Line A, stomach carcinomata originally obtained from the Animal Supply and Research Units of the British Empire Cancer Campaign) now in its 83rd generation transplant in our laboratory.

Biphenyl-4,4′-di-(sulfonamidobenzene-S^{35}-4-sulfonamide) (III) was studied in 8 additional animals: 2 mice 4·5 weeks old with 4-day-old tumor transplant, 2 mice 7 weeks old with 3-week-old tumor transplant, 2 mice 8·5 weeks old from which a 4-week-old tumor had been excised 3 days prior to receiving the compound, and 2 mice 11 weeks old from which a 4-week-old tumor had been removed 3 weeks prior to the study (Table V). Four other mice were pylorus-ligated according to the method previously described (Klein, Argus, and Ray, 1953) prior to receiving biphenyl-4,4′-di-(sulfonamidobenzene-4-carboxylic acid)-S^{35} (II).

The sulfur 35-labeled compounds were administered by tail vein in the following amounts per mouse: fluorene-2,7-di-(sulfonamidobenzene)-S^{35} (I), 3 mg. in 0·5 ml. of 0·05 N sodium hydroxide; biphenyl-4,4′-di-(sulfonamidobenzene-4-carboxylic acid)-S^{35} (II), 4 mg. in 0·5 ml. of 0·05 N sodium bicarbonate; and biphenyl-4,4′-di-(sulfonamidobenzene-S^{35}-4-sulfonamide) (III), 4 mg. in 0·5 ml. of 0·05 N sodium bicarbonate. Animals were sacrificed at 2-, 8-, 16-, and 32-hour intervals following treatment, and pooled samples from 2 animals were used for each determination. Just prior to sacrifice, the mice were anesthetized with nembutal, and blood was removed by heart puncture using a heparinized syringe. The blood was centrifuged to separate plasma and cells. Plasma was plated using 0·4 ml. plasma plus 0·6 ml. 1 per cent sodium hydroxide per planchet. The cells were washed twice with saline and planchets were plated with 0·3 ml. cells plus 0·7 ml. 1 per cent sodium hydroxide. The tissues to be studied were removed, weighed immediately and placed in 10 ml. of 1 per cent sodium hydroxide (containing 1 ml. of Tergitol wetting agent per 250 ml.) per gram of tissue. Excreta were suspended in sufficient water to bring the total volume to 100 ml., and 20 ml. of 5 per cent sodium hydroxide was added to this. After storing at 5° for 48 hours, the tissues were solubilized by autoclaving at 15 lb. for 30 minutes and a 1 ml. aliquot of each was plated on a stainless steel planchet.

Radioactivity measurements were made with a Nuclear-Chicago, Model D-47, thin window, Q-gas counting chamber with Model C-110 automatic sample changer and Model 183 scaler. The efficiency was 20 per cent and duplicate sample counts were within 3 per cent. The concentration of the sulfur 35-labeled disulfonamides in each sample was determined by direct comparison with standard planchets prepared in the same manner and containing known concentrations of the radioactive compounds. To eliminate correction for the decay of $sulfur^{35}$, the standards were counted the same day as the samples. Total blood volume was calculated on the basis of 63·2 ml. per kg. body weight (Oakley and Warrack, 1940).

RESULTS AND DISCUSSION

The distribution of radioactivity in tumor-bearing and tumor-free CAF_1/Jax mice following intravenous injection of fluorene-2,7-di-(sulfonamidobenzene)-S^{35} (I) is given in Table I. These data show that 2 hours after administration, the concentration of compound in the tumor is less than in any other tissue. At 8 hours, however, the concentration of radioactivity decreases in all normal tissues except the large intestine, but the value for the neoplasm remains essentially the same as the 2-hour value. The concentration of radioactivity in the tumor at 8 hours exceeds that in blood cells and plasma, spleen, kidneys, muscle and carcass. The level of radioactivity in the gastrointestinal tract is high at all time intervals studied. The ratios of the concentration of this compound in tumor tissue to that in the blood, vital organs, skin, muscle and carcass are tabulated in Table II. Values greater than unity indicate favorable tumor localization.

TABLE I.—*Distribution of Radioactivity in Mice at Different Time Intervals Following Intravenous Injection of Fluorene-2,7-di-(sulfonamidobenzene)-S^{35}*

	Concentration in μg. compound per g. tissue, or ml. blood cells, or plasma				Percentage administered radioactivity recovered			
	2 hr.*	8 hr.†	16 hr.‡	32 hr.‡	2 hr.*	8 hr.†	16 hr.‡	32 hr.‡
Tumor-bearing animals:								
Blood cells	37	4·2	0·3	0·2	0·92	0·09	0·81	0·45
Blood plasma	107	7·5	2·2	1·1	2·65	0·15	5·91	2·50
Liver	325	131·5	53·5	29·3	14·98	4·41	2·70	1·23
Lungs	125	51·5	21·5	13·5	0·47	0·18	0·11	0·07
Spleen	56	21·5	8·2	6·3	0·36	0·11	0·06	0·03
Kidney	105	25·5	10·7	7·5	1·16	0·25	0·14	0·08
Skin	76	56·0	12·0	16·7	9·72	6·21	1·67	1·84
Leg muscle	63	19·0	6·0	4·0	0·29	0·10	0·05	0·03
Stomach plus contents	137	64·0	20·0	50·0	0·95	0·36	0·13	0·28
Small intestine plus contents	470	173·0	58·0	70·0	21·86	6·86	2·68	2·98
Large intestine plus contents	880	2705·0	1513·0	600·5	4·84	54·73	41·81	13·03
Carcass	52	12·0	6·8	4·0	21·10	3·82	2·90	1·32
Excreta	—	—	—	—	3·67	8·34	24·28	45·00
Tumor	36	35·5	28·8	18·2	0·33	0·24	0·35	0·23
Total	—	—	—	—	83·30	85·85	83·60	69·07
Tumor-free animals:								
Blood cells	46·0	5·0	0·3	0·3	1·06	0·13	0·66	0·72
Blood plasma	155·5	6·0	2·7	2·0	3·54	0·17	5·93	4·81
Liver	273·5	118·0	59·7	33·0	12·69	5·24	2·14	1·27
Lungs	130·5	15·5	19·2	13·2	0·55	0·07	0·08	0·05
Spleen	70·0	11·5	10·5	7·0	0·49	0·37	0·03	0·02
Kidney	115·0	16·0	14·0	9·5	1·30	0·21	0·15	0·10
Skin	102·0	37·0	18·7	11·2	13·01	5·27	2·07	1·14
Leg muscle	94·5	10·5	6·3	4·3	0·50	0·06	0·04	0·04
Stomach plus contents	85·0	203·0	40·0	90·0	0·96	1·87	0·22	0·68
Small intestine plus contents	510·0	145·0	73·0	112·0	25·40	7·28	2·85	4·85
Large intestine plus contents	435·0	1887·5	1868·0	760·0	13·71	53·84	43·13	15·00
Carcass	60·5	7·5	9·2	6·8	23·20	3·19	3·23	2·39
Excreta	—	—	—	—	3·32	7·43	15·55	45·11
Total	—	—	—	—	99·73	85·13	76·08	76·18

* Average value from 2 animals.
† Average value from 4 animals.
‡ Average value from 6 animals.

TABLE II.—*Ratios of the Concentration of Radioactiity in Tumor Tissue to the Concentration in Other Tissues Following a Single Injection of Fluorene-2,7-di-(sulfonamidobenzene)-S^{35} to Tumor-bearing Mice**

	Time interval following injection			
	2 hr.	8 hr.	16 hr.	32 hr.
Blood cells	0·97	7·45	96·00	91·00
Blood plasma	0·34	4·73	13·10	16·55
Liver	0·11	0·27	0·54	0·62
Lungs	0·29	0·69	1·34	1·35
Spleen	0·64	1·65	3·51	2·89
Kidney	0·34	1·39	2·69	2·43
Skin	0·47	0·63	2·38	1·09
Leg muscle	0·57	1·87	4·80	4·55
Carcass	0·69	2·96	4·24	4·55

* A ratio greater than unity is favorable.

Thus, after 16 hours there is an increase in ratio in every case over the 8-hour values. Only the liver localizes the compound to a greater extent than does the tumor. At 32 hours the tumor still concentrates more of the radioactive material than do the other tissues with the exception of the liver. One can conclude, for this compound, that except for the liver the localization by tumor tissue compares favorably with that of disodium fluorene-2,7-disulfonate-S^{35}. The latter gives a larger ratio for the liver, but concentrates more in the kidneys and gastrointestinal tract than in neoplastic tissue (Argus, 1953). Another sulfonated compound of this type, fluorene-2,7-di-(sulfonamido-S^{35}-2′-naphthalene-X-sulfonic acid) shows a greater localization in tumor tissue at 32 hours than in any other tissue studied (Argus and Hewson, 1954). Thus, the introduction of sulfonic acid groups into the molecule seems to enhance tumor affinity. The possibility that sulfonation might increase the activity of compound I will be studied in a later phase of these investigations.

In Table III is summarized the distribution of radioactivity following administration of the two disulfonamido derivatives of biphenyl. The compound having two free carboxy groups, biphenyl-4,4′-di-(sulfonamidobenzene-4-carboxylic acid)-S^{35} (II), localizes in tumor tissue to a greater extent than in blood cells, spleen, and leg muscle at 2 hours. The concentration in the tumor at 8 hours is the same or greater than that in all other tissues, except the gastrointestinal tract. The high level of this compound in the stomach contents, 2 hours following intravenous injection, suggested possible secretion by the stomach wall. To investigate this, four pylorus-ligated mice were injected intravenously with sulfur 35-labeled compound. For two animals sacrificed at 2 hours, the average recovery of radioactivity from the stomach contents is 0·75 per cent of the administered dose. At 8 hours the average recovery is 2·13 per cent. This secretion of a highly acid compound is not in agreement with the results of Ingraham and Visscher (1935) on the gastric secretion of dyestuffs. These authors found that only basic dyes are secreted by the gastric glands of the dog. Previous work in this laboratory, however, has indicated that an acid dye can be secreted by the rat stomach (Cambel, Breidenbach and Ray, 1954).

Biphenyl-4,4′-di-(sulfonamidobenzene-S^{35}-4-sulfonamide) (III) does not show selective tumor affinity. Thus, all body constituents except the blood plasma,

TABLE III.—*Distribution of Radioactivity in Mice Following Intravenous Injection of Disulfonamido Derivatives of Biphenyl*

	Concentration in μg. compound per g. tissue, or ml. blood cells, or plasma*				Percentage administered radioactivity recovered*			
	Compound II†		Compound III‡		Compound II†		Compound III‡	
	2 hr.	8 hr.	2 hr.	8 hr.	2 hr.	8 hr.	2 hr.	8 hr.
Tumor-bearing animals:								
Blood cells	<10·0	<10·0	94·0	33·0	<0·001	<0·001	1·44	0·44
Blood plasma	38·5	10·0	268·0	20·0	0·84	0·23	6·16	0·42
Liver	82·5	17·5	450·0	82·0	2·44	0·48	14·34	2·25
Lungs	41·5	13·0	254.0	72·0	0·08	0·03	0·67	0·19
Spleen	9·0	3·0	62·0	15·0	0·04	0·01	0·28	0·06
Kidney	42·5	9·0	644·0	76·0	0·30	0·06	4·98	0·50
Skin	102·5	10·0	238·0	28·0	0·22	0·03	0·68	0·05
Leg muscle	14·0	0·0	58·0	10·0	0·04	0·00	0·24	0·04
Stomach plus contents	308·0	41·5	155·0	296·0	1·54	0·18	0·92	1·20
Small intestine plus contents	1129·0	89·0	492·0	252·0	33·76	2·44	19·92	11·48
Large intestine plus contents	1405·0	2410·0	510·0	2050·0	21·58	35·73	10·44	36·50
Carcass	36·5	10·0	95·0	18·0	14·04	3·94	37·82	5·84
Excreta	—	—	—	—	15·22	35·57	6·40	28·40
Tumor	30·0	17·5	60·0	25·0	0·49	0·38	0·41	0·16
Total	—	—	—	—	90·59	79·08	104·70	87·53
Tumor-free animals:								
Blood cells	<10·0	<10·0	112·0	40·0	<0·001	<0·001	1·55	0·45
Blood plasma	39·5	10·5	370·0	106·0	0·84	0·24	7·79	1·86
Liver	97·5	12·5	402·0	142·0	2·56	0·34	11·44	3·27
Lungs	85·5	46·5	411·0	74·0	0·22	0·14	1·33	0·18
Spleen	10·0	4·0	128·0	35·0	0·02	0·01	0·28	0·04
Kidney	46·5	8·0	1022·0	572·0	0·36	0·06	7·06	4·01
Skin	110·0	12·5	298·0	100·0	0·24	0·03	0·80	0·16
Leg muscle	16·5	0·0	88·0	22·0	0·06	0·00	0·34	0·08
Stomach plus contents	274·0	54·0	142·0	78·0	1·27	0·22	1·06	0·33
Small intestine plus contents	844·0	114·0	361·0	345·0	26·60	3·16	16·24	13·96
Large intestine plus contents	1000·0	2085·0	235·0	1734·0	20·94	41·22	5·04	32·48
Carcass	28·0	11·5	128·0	42·0	11·18	4·80	46·60	13·18
Excreta	—	—	—	—	12·41	23·45	5·18	29·62
Total	—	—	—	—	76·70	73·67	104·71	99·62

* Each value is the average from 4 animals.
† Biphenyl-4,4′-di-(sulfonamidobenzene-4-carboxylic acid)-S^{35}.
‡ Biphenyl-4,4′-di-(sulfonamifobenzene-S^{35}-4-sulfonamide).

spleen, leg muscle and carcass concentrate more radioactive material at 8 hours than does the tumor (Table III). In previous studies, another compound that failed to localize in tumor tissue, fluorene-2,7-di-(sulfonamido-2′-naphthalene)-S^{35}, did concentrate in the liver and spleen of tumor-free CAF_1/Jax mice to a greater extent than in these organs of mice bearing a transplanted squamous cell tumor (Argus and Hewson, 1954). The possible value of this phenomenon in cancer diagnosis led to more extensive studies which showed that difference in uptake of

this compound exists also in other strains and species of animals, and with various types of transplanted tumors (Argus, Hewson and Ray, 1956; Argus, Lemasters, Gutierrez and Ray, 1957; and Argus, Seepe, Kane and Ray, 1958). Since biphenyl-4,4′-di-(sulfonamidobenzene-S^{35}-4-sulfonamide) (III) did not show selective tumor localization, differences in the uptake of this compound by the tissues of tumor-free and tumor-bearing mice were investigated.

The ratios of the concentration of radioactivity in the liver, spleen, kidneys and blood plasma of tumor-free mice to the concentration in these tissues of tumor-bearing mice following administration of biphenyl-4,4′-di-(sulfonamidobenzene-S^{35}-4-sulfonamide) (III) are given in Table IV. The liver of tumor-free mice

TABLE IV.—*Ratios of Concentration of Radioactivity in the Tissues of Tumor-free Mice to the Concentration in Tissues of Tumor-Bearing Mice at 2 and 8 hours following Intravenous Injection of Biphenyl-4,4′-di-(sulfonamidobenzene-S^{35}-4-sulfonamide)*

	Ratio*	
	2hr.	8 hr.
Liver . . .	0·89	1·73
Spleen . . .	2·06	2·33
Kidneys . . .	1·59	7·53
Blood plasma . .	1·38	5·30

$$*\left(\frac{\text{Concentration in tissue of tumor-free mice}}{\text{Concentration in tissue of tumor-bearing mice}}\right);$$

Concentration is expressed as μg. compound/g. of tissue or ml. blood plasma; each concentration value is the average from 4 animals.

localizes this compound in greater concentration than does the liver of tumor-bearing mice at 8 hours, but not at 2 hours. The phenomenon is present for the spleen at both time intervals studied. At 8 hours this ratio for the liver is about the same as for fluorene-2,7-di-(sulfonamido-2′-naphthalene)-S^{35} in CAF_1/Jax mice; the ratio of the spleen is, however, less than that for the fluorene derivative (Argus, Hewson and Ray, 1956).

With biphenyl-4,4′-di-(sulfonamidobenzene-S^{35}-4-sulfonamide) (III) there are two features not found with previous compounds. These are the large differences in localization in the kidneys and in the blood plasma of tumor-free and tumor-bearing animals. Thus, at 8 hours the kidneys of control animals localize 7·53 times as much radioactive material as do the kidneys of tumor-bearing animals (Table IV). This difference in the level of compound in the kidneys is not reflected in the rate of excretion (Table III), since the percentage of administered dose recovered from the excreta is approximately equal for tumor-free and tumor-bearing animals at 2 and at 8 hours.

Two hours after intravenous injection of biphenyl-4,4′-di-(sulfonamidobenzene-S^{35}-4-sulfonamide) (III) the concentration of radioactivity in the blood plasma of tumor-free mice is 1·38 times that in the plasma of tumor-bearing mice. At 8 hours the ratio increases to 5·30. Since the difference is measurable in a readily accessible body fluid, this phenomenon may have greater promise as a diagnostic tool. Further studies were therefore carried out at different times following tumor transplantion and tumor excision. The ratios obtained for the blood plasma 2 hours after administration of the biphenyl compound are shown in Table V. Four

TABLE V.—*Concentration of Biphenyl-4,4′-di-(sulfonamidobenzene-S^{35}-4-sulfonamide) in the Blood Plasma of Tumor-free, Tumor-bearing, and Tumour-excised CAF_1/Jax Mice 2 hours after Intravenous Injection*

Mice	Concentration*	Ratio†
Tumor-free	370‡	1·00
Bearing tumor 4 days	354§	1·05
Bearing tumor 3 weeks	290§	1·28
Bearing tumor 4 weeks	268‡	1·38
Three days after tumor excision¶	231§	1·60
Three weeks after tumor excision¶	330§	1·12

* μg. compound/ml. plasma.

† $\left(\frac{\text{Concentration in tumor-free plasma}}{\text{Concentration in tumor-bearing plasma}}\right)$.

‡ Average value from 4 animals.

§ Average value from 2 animals.

¶ Tumor excised 4 weeks after transplantation.

days after transplantation a slight increase in the ratio is noted; at 3 weeks the ratio increases to 1·28; at 4 weeks the ratio is 1·38. Tumor excisions were made at 4 weeks following transplantation; no metastases had occurred prior to excision of the tumor. The ratio is still high (1·60) 3 days after excision, indicating that the animals have not yet begun to return to normal; by 3 weeks the ratio drops to 1·12. These ratios indicate that the capacity of blood plasma to concentrate this biphenyl disulfonamide is influenced by the presence or absence of a tumor in the animal body.

In conclusion, previous and present studies on this subject indicate that at least two factors are important for tumor localization: (*a*) an optimum size of the molecule, and (*b*) the presence of strong acidic groups. Thus, of fluorene-2,7-disulfonamide-S^{35}, fluorene-2,7-di-(sulfonamidobenzene)-S^{35}, and fluorene-2,7-di-(sulfonamido-2′-naphthalene)-S^{35}, only fluorene-2,7-di-(sulfonamidobenzene)-S^{35}, the compound of intermediate size, shows selective tumor localization. However, by introducing sulfonic acid groups into the naphthalene moiety of fluorene-2,7-di-(sulfonamido-2′-naphthalene)-S^{35}, the compound acquires tumor affinity, Similarly fluorene-2,7-disulfonic acid-S^{35} shows selective localization. This enhancing ability of acidic groups in the molecule is also evident in comparing the tumor localization of the two derivatives of biphenyl investigated here. Thus, biphenyl-4,4′-di-(sulfonamidobenzene-4-carboxylic acid)-S^{35} shows higher tumor localization ratios than biphenyl-4,4′-di-(sulfonamidobenzene-S^{35}-4-sulfonamide).

While studies with fluorene-2,7-di-(sulfonamido-2′-naphthalene)-S^{35} have shown that the presence of a tumor in the animal body affects the ability of the spleen and liver to localize the compound, the present work with biphenyl-4,4′-di-(sulfonamidobenzene-S^{35}-4-sulfonamide) suggests that the presence of a neoplasm also may influence the ability of the blood and kidneys to concentrate certain large molecular weight compounds. What seems to be of importance, from the diagnostic standpoint, is that a difference in the localizing ability of a tissue in tumor-free and tumor-bearing animals has been demonstrated in a readily accessible body constituent.

SUMMARY

1. Syntheses are described for fluorene-2,7-di-(sulfonamidobenzene)-S^{35}, biphenyl-4,4′-disulfonyl chloride-S^{35}, biphenyl-4,4′-di-(sulfonamidobenzene-4-

carboxylic acid)-S^{35} and biphenyl-4,4′-di-(sulfonamidobenzene-S^{35}-4-sulfonamide).

2. The distribution of radioactivity in tumor-bearing and tumor-free mice following intravenous injection of the three disulfonamides showed fluorene-2,7-di-(sulfonamidobenzene)-S^{35} to have the most favorable tumor localization.

3. Experiments with biphenyl-4,4′-di-(sulfonamidobenzene-4-carboxylic acid)-S^{35} in pylorus-ligated mice revealed this acid compound to be secreted by the gastric mucosa.

4. Biphenyl-4,4′-di-(sulfonamidobenzene-S^{35}-4-sulfonamide) is concentrated by the liver, spleen, kidneys and blood plasma of tumor-free mice to a greater extent than by these organs of tumor-bearing mice, thus revealing diagnostic possibilities.

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THE INTERMEDIARY METABOLISM OF 3 : 4-BENZPYRENE : THE BIOSYNTHESIS AND IDENTIFICATION OF THE X_1 AND X_2 METABOLITES

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A PREVIOUS study of the intermediary metabolism of 3 : 4-benzpyrene has been reported (Harper, 1958*b*). The X_1 metabolite (BPX_1) was considered to be the glucuronide conjugate of F_1, to which a free phenolic nature was assigned, and evidence was obtained that 8-benzpyrenol is also eliminated in conjugated form in the bile. The X_2 metabolite (BPX_2) was not identified but its absorption spectrum was considered to be more in keeping with a fully aromatic benzpyrenoid configuration rather than the dihydroxy-dihydro structure proposed by Weigert and Mottram (1946).

Conjugation with glucuronic and sulphuric acids is known to be a general process for the detoxification of phenols. It seemed hopeful therefore that reintroduction of 8-benzpyrenol and the F_1 phenol into the animal body would lead to their elimination in the bile or urine in conjugation with these two acids. These conjugates could then be used as reference compounds for the fractionation and subsequent identification of the intermediate metabolites of 3 : 4-benzpyrene.

MATERIALS AND METHODS

8-Benzpyrenol was synthesised by the method of Cook, Ludwiczak and Schoental (1950). Final purification was effected by chromatography on alumina from benzene followed by crystallisation from benzene-light petroleum. The resulting yellow needles melted at 225–228° C. (decomp.) and spectroscopically appeared almost identical to 8-benzpyrenol of metabolic origin (Berenblum, Crowfoot, Holiday and Schoental, 1943; Weigert and Mottram, 1946).

The absorption maxima (in ethanol) were at 228, 259, 268, 293–294, 308, 346, 362, 380·5, 402 and 426 mμ. There is a slight displacement of these figures from those reported by Conney, Miller and Miller (1957) for synthetic 8-benzpyrenol in ethanol.

The phenol was prepared for injection as a fine colloidal dispersion containing 0·5 mg. per c.c. by dissolving it in a small volume of acetone and injecting the acetone solution beneath the surface of glass double-distilled water. The acetone was then pumped off *in vacuo*. The mice (Strong A strain) in batches of 10–15 received an intravenous injection of 0·5 c.c. of this colloid and were then killed at intervals of 1–4 hours.

The F_1 phenol was obtained by acid hydrolysis of pooled X_1 metabolite isolated from the small intestines of mice which had been injected with 3 : 4-benzpyrene. It was prepared as a colloidal dispersion as described for 8-benzpyrenol and this was used for the injection of four mice.

After the desired time interval the particular organ to be examined was quickly dissected out and subjected to an extraction procedure similar to that described for the intermediate metabolites of 3 : 4-benzpyrene. This involved initial extraction with 70 per cent acetone, removal of the acetone *in vacuo* and saturation of the aqueous phase with ammonium sulphate. After repeated extraction with xylene the aqueous phase was then acidified with hydrochloric acid and re-extracted with xylene. This resulted in the transference of all blue fluorescent material into the xylene phases. These were then combined and chromatographed on columns of silica gel for the removal of possible X-type derivatives. Free phenol and quinone in the filtrate from this adsorbent were detected by chromatography on alumina.

All descriptions of fluorescence refer to the appearance under ultra-violet light.

β-Glucuronidase (bacterial) and *p*-nitrophenylsulphate were obtained from the Sigma Chemical Company, Missouri, potassium hydrogen saccharate from George T. Gurr Ltd., silica gel (100/200 mesh), adenylic acid, α-ketoglutaric acid and adenosinetriphosphate (ATP) from L. Light and Co. Ltd. and alumina from B.D.H. Ltd. Takadiastase was a gift from Parke Davis, Co. Ltd.

All organic reagents were obtained free from fluorescence by fractional distillation.

RESULTS

Metabolism of 8-benzpyrenol

The appearance of the opened abdominal cavity under ultra-violet light was very similar to that arising from the intravenous injection of the parent hydrocarbon, 3 : 4-benzpyrene. A strong blue fluorescence was associated with the gall bladder, gut and kidney but the weak fluorescence of the bladder suggested that little excretion of fluorescent metabolities was taking place in the urine.

(a) *Duodenum and small intestine.*—Chromatography of the xylene extract on silica gel yielded a green coloured, strong blue/white fluorescent zone at the surface. On development with amyl alcohol a strong blue fluorescent fraction passed down the column and was collected in the filtrate, the initial yellow non-fluorescent fraction being discarded.

The amyl alcohol filtrate was diluted with 10 times its own volume of petroleum ether (b.p. 40–60° C.) and rechromatographed on silica gel. The bright blue fluorescent metabolite was held as an almost colourless zone at the surface from which it was readily eluted with ethanol. The absorption spectrum of the eluate was similar to, but not identical with, the X_1 metabolite derived from 3 : 4-benzpyrene (Fig. 1, Table I).

After development with amyl alcohol the whole of the original silica gel column possessed a blue/white fluorescence, this being most intense in the green coloured surface zone. On elution with ethanol this surface zone yielded a pale green solution with an intense blue fluorescence. Its absorption spectrum was similar to that of X_2 derived from 3 : 4-benzpyrene but it obviously contained strongly absorbing background material. Partial purification was effected by transfer to water, acidification with hydrochloric acid and extraction with ether. The metabolite passed out in the ether phase and possessed the spectrum shown (Fig. 2, Table I).

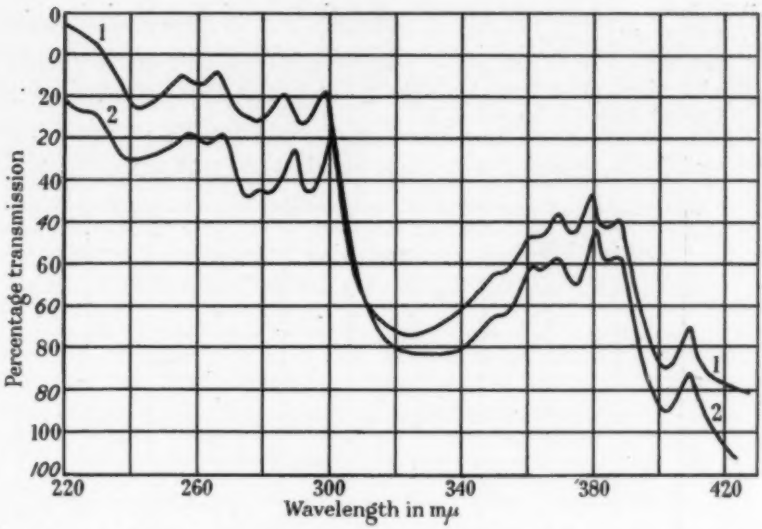

FIG. 1.—Absorption spectra in ethyl alcohol. 1. X_1 metabolite of 3 : 4-benzpyrene (upper scale). 2. X_1 metabolite of 8-benzpyrenol (lower scale).

A trace amount only of free 8-benzpyrenol was detected on the alumina chromatogram.

TABLE I

	Absorption maxima in ethanol—mμ									
BPX_1 . .	256	266	—	286	298	363	369	379	388	408–409
8-OH.X_1 .	256–257	268	279	289	302	362	369	381	388	409
F_1X_1 . .	—	—	—	—	298	362	369	378–380	—	406–410
						inf.	inf.			inf.
BPX_2 . .	256–260	268	—	287	298	362–364	—	380	392	414
8-OH.X_2 .	256	268	—	290	303	362	—	381	392	416–417

BPX_1 and BPX_2 = X_1 and X_2 metabolites of 3 : 4-benzpyrene.
8-OH.X_1 and 8-OH.X_2 = X_1 and X_2 metabolites of 8-benzpyrenol.
F_1X_1 = X_1 Metabolite of the F_1-phenol.

(b) *Caecum and large intestine.*—Extracts from animals killed 1 hour after injection yielded only small amounts of the X-type derivatives and free 8-benzpyrenol. The amount of free phenol increased progressively with time and a considerable quantity was present 4 hours after injection.

(c) *Bile.*—Extraction of the bright blue fluorescent bile with ether removed little fluorescent material but after acidification an ether extract possessed a bright blue fluorescence. After transfer to xylene and chromatography on silica gel followed by alumina X_1- and X_2-type derivatives were identified on the silica gel and free 8-benzpyrenol on the alumina. The extraction of free phenol only after acidification suggested its formation from an acid-labile precursor. This factor will be referred to later.

(d) *Kidney.*—Appreciable amounts of both X_1- and X_2-type derivatives were obtained from this tissue.

(e) *Bladder.*—A small amount of 8-benzpyrenol was extractable directly from the urine by ether. Only small amounts of the X-type derivatives and free phenol were extracted after acidification.

(f) *Liver.*—Little blue fluorescent material was extracted with 70 per cent acetone and the X_1-type derivative only was identified with any certainty.

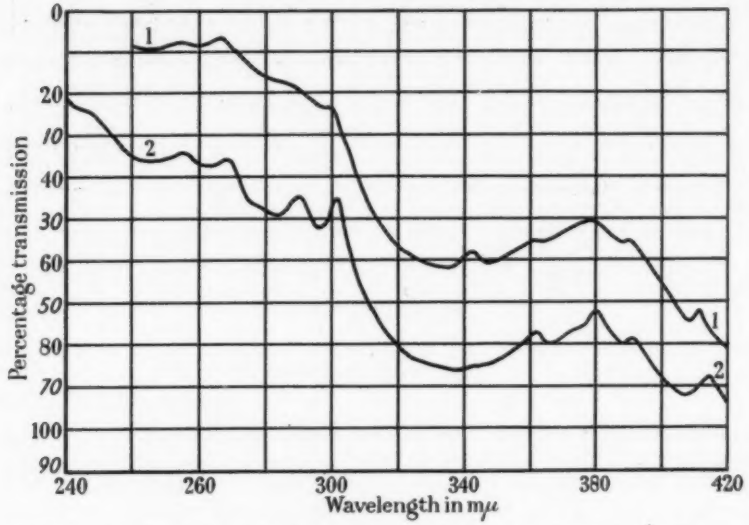

FIG. 2.—Absorption spectra in ethyl alcohol. 1. X_2 metabolite of 3 : 4-benzpyrene (upper scale). 2. X_1 metabolite of 8-benzpyrenol (lower scale).

Properties and identification of the metabolites

The physical and chemical properties of the two derivatives closely paralleled those previously reported for the X-metabolites of 3 : 4-benzpyrene (Weigert and Mottram, 1946; Harper, 1958*b*).

Both were adsorbed on silica gel from xylene and the X_1-type only was eluted with amyl alcohol. The X_2-type was associated with the coloured surface zone of the chromatogram and was only partially eluted with ethanol. Better elution was effected with either 1 : 1 ethanol-pH 6 phosphate buffer or ethanol containing a few drops of hydrochloric acid.

Both were strongly adsorbed on alumina and the X_1-type only was partially removed by ethanol.

Both were water soluble although a certain amount of insoluble matter remained on evaporating the ethanolic eluates to dryness.

On gently warming in 0·1 N-HCl under nitrogen the X_1-type was rapidly converted to 8-benzpyrenol whereas the X_2-type remained unaffected. With stronger acid (10 N-HCl) and heating the solution at 100° C. for 2 hours under nitrogen the X_2-type was also converted to 8-benzpyrenol.

Further investigation of the hydrolytic conditions revealed that, after the addition of acid to an aqueous solution of the X_1-type metabolite, although there was no apparent change in its absorption spectrum, a considerable amount of free 8-benzpyrenol was immediately extractable with ether. It appeared therefore that the metabolite was instantly decomposed by the combined effect of acid and ether.

The most likely explanation for this behaviour was that the metabolites were sulphate and glucuronide conjugates of 8-benzpyrenol for, in an analogous study of the metabolism of 1-naphthol (Boyland and Wiltshire, 1953), the phenol was excreted either as free naphthol, naphthylsulphuric acid or naphthylglucuronide and only a relatively small amount was degraded by metabolic processes in the animal body. The evidence thus far pointed towards the X_1-type derivative being the sulphate and the X_2-type the glucuronide for phenylglucuronides are, in general, more resistant to acid hydrolysis than are the sulphates (Bray and Thorpe, 1954) and, in the case of steroid conjugates, the glucuronides are more strongly adsorbed on alumina than are the sulphates (Barlow, 1957).

Experiments were therefore carried out to test this hypothesis.

Test for the glucuronide moiety

Solutions of the metabolites in 2 c.c. water (estimated as containing as much as 25 γ) were heated with 2 c.c. 0·375 per cent naphthoresorcinol reagent and 4 c.c. technical hydrochloric acid at 100° C. for 2 hours. The mixtures were then cooled and extracted with 10 c.c. ether. The ether extract of the X_1-type was pale yellow in colour although a reddish tinge was given by impure samples of the metabolite contaminated by yellow colouring matter. The X_2-type invariably yielded a reddish/blue colour.

Although not conclusive this suggested a glucuronide moiety in the X_2-type derivative.

Action of β-glucuronidase

Detailed studies of the enzyme β-glucuronidase and of its inhibition by saccharo-1 : 4-lactone have been made by Levvy and his co-workers (Levvy, 1948, 1952; Karunairatnam and Levvy, 1949) and the statement is made (Conchie and Levvy, 1957) that saccharo-1 : 4-lactone as such, or in the form of a saccharate solution, has been shown to inhibit β-glucuronidase from every source that has been studied.

Incubations of the metabolites with β-glucuronidase were carried out as described in the Sigma Bulletin No. 105 using a 1 per cent solution of the enzyme. In control experiments boiled 1 per cent solutions were used. The incubates were then cooled and extracted with ether.

The extracts from the control and X_1-type incubates were only weakly fluorescent whereas that from the X_2-type was bright blue and exhibited the characteristic spectrum of 8-benzpyrenol.

In the presence of 10^{-2} M boiled saccharate solution the hydrolytic effect of the enzyme on the X_2-type metabolite was almost completely abolished.

This result indicated a β-glucuronosidic linkage in the X_2-type derivative only.

Action of takadiastase

A detailed investigation of the arylsulphatase activity of takadiastase was made by Robinson, Smith, Spencer and Williams (1952).

The optimum pH for arylsulphatase activity appears to be about 6 but in the present experiments acetate buffer pH 6·8 was used in order to eliminate possible pH effect on the metabolites. Under these conditions the takadiastase still possessed appreciable activity towards *p*-nitrophenylsulphate as substrate but the activity was bolished on boiling the enzyme solution for 15 minutes.

Incubations were carried out as described by Robinson *et al.* using the boiled enzyme as control. The incubates were then cooled and extracted with ether.

The extracts from the control and X_2-type incubates were only weakly fluorescent whereas that from the X_1-type was bright blue and exhibited the characteristic spectrum of 8-benzpyrenol.

An arylsulphate linkage was thus indicated in the X_1-type metabolite only.

Liver slice experiments

The conjugation of phenols with sulphuric and glucuronic acids in isolated liver slices has been reported by Storey (1950), De Meio and Tkacz (1952).

Fifteen grammes mouse liver slices were incubated in 50 c.c. Tyrode buffer containing 1 mg. 8-benzpyrenol with oxygenation. The slices soon assumed a bright blue fluorescence and after 2 hours were extracted as described for the *in vivo* tissue.

Appreciable amounts of both X_1- and X_2-type derivatives and benzpyrene-5 : 8-quinone were identified on the chromatograms in addition to unchanged 8-benzpyrenol.

Glucuronide synthesis in liver homogenate

The failure of liver homogenates to synthesise glucuronides was studied by Dutton and Storey (1954). They found that glucuronide synthesis could be restored by the addition of an extract of boiled liver to the homogenate and subsequent work led to the identification of the essential factor as being uridine disphosphate glucuronic acid (UDPGA) (Storey and Dutton, 1955).

An investigation of the metabolism of 8-benzpyrenol in mouse liver homogenate was first made under the conditions described for liver slices. Under these conditions a small amount of the X_1-type derivate only was identified on the silica gel chromatogram. Free phenol and 5 : 8-quinone were obtained on the alumina column.

Experiments were then made with liver homogenate fortified by the addition of crude UDPGA prepared from guinea-pig liver (Dutton and Storey, 1954).

A 10 per cent mouse liver homogenate was prepared in 0·154 M-KCl containing $3{\cdot}2 \times 10^{-4}$ M $KHCO_3$. Incubations were then carried out at 37° C. with 30 c.c. homogenate to which was added 20 c.c. 0·2 M phosphate buffer pH 7·4, 25 mg. ascorbic acid in 12 c.c. water, 6 c.c. 0·3 M $MgCl_2$, 5 c.c. of colloidal 8-benzpyrenol containing 1·25 mg., 35 c.c. of crude UDPGA extract and 50 c.c. water. In a control experiment the UDPGA extract was substituted by 35 c.c. water. After 2 hours the incubates were extracted as described for the *in vivo* tissue.

The extract of the fortified homogenate yielded a wide bright blue fluorescent zone at the surface of the silica gel which was absent from the control column.

Little blue fluorescent material was removed from this zone on prolonged development with amyl alcohol. On elution with ethanol it was identified as the X_2-type metabolite by its conversion to 8-benzpyrenol both by β-glucuronidase and prolonged acid hydrolysis.

Considerable amounts of the 5 : 8-quinone were obtained from both the experimental and control incubates, possibly due to oxidation of the phenol by ascorbic acid (cf. Warren, 1943).

It is concluded therefore that formation of the X_2-type metabolite is dependent upon the presence of UDPGA and in this respect conforms to accepted knowledge concerning the synthesis of glucuronides.

Sulphate synthesis in liver homogenate

Following on from preliminary work on the synthesis of sulphates in liver homogenate (De Meio and Tkacz, 1950, 1952; De Meio and Wizerkaniuk, 1952; De Meio, 1952) it was shown by Bernstein and McGilvery (1952) and De Meio, Wizerkaniuk and Fabiani (1953) that the enzyme system is located in the high speed supernatant fraction of the homogenate and requires ATP and Mg^{++} ions for activity.

In the present experiments a 10 per cent mouse liver homogenate in Tyrode buffer (60 c.c.) was fortified with 20 c.c. 0·154 M $MgSO_4$ and 10 mg. ATP (sodium salt) and incubated with 0·5 mg. of 8-benzpyrenol at 37° C. for 2 hours. In a control expriment ATP was omitted from the mixture.

After extraction the X_1-type derivative only was identified with any certainty in the control experiment. The ATP fortified homogenate however, yielded a much increased yield of the X_1-type derivative and a smaller zone of what appeared to be the X_2-type. On elution with ethanol however, this latter fraction exhibited predominantly the absorption spectrum of the X_1-type metabolite suggesting that, in the absence of much X_2-type derivative, the X_1-type is only partially removed from silica gel by amyl alcohol. This provides a possible explanation for the appearance of the chromatogram noted for tissue extracts of animals injected with 8-benzpyrenol (this paper) and 3 : 4-benzpyrene (Harper, 1958*b*).

After elution with ethanol, further blue fluorescent material was removed from the X_2-type zone by 1 : 1 ethanol-phosphate buffer but insufficient was obtained for characterisation.

Contrary to the findings of De Meio, Wizerkaniuk and Fabiani (1953) with respect to the synthesis of phenyl-sulphate in liver homogenate, replacement of the ATP by a mixture of adenylic and α-ketoglutaric acids did not increase the yield of the X_1-type metabolite.

Experiments using fortified " microsome free " supernatant fractions of the homogenate have given varied results. An initial preparation gave excellent yields of the X_1-type metabolite only. In repetitive experiments however, the account of the X_1-type derivative varied considerably being practically zero on occasions. No explanation for this behaviour can be offered but it is possibly due to technical errors inherent in the establishment of the *in vitro* sulphate synthesising system.

No definite conclusion can therefore be arrived at from these experiments but it is considered significant that, on the occasions when evidence of increased metabolism has been obtained, the X_1-type derivative has been present as the major metabolite.

Metabolism in cat liver slices

Certain evidence exists to suggest that cat liver does not synthesise glucuronides (Hartiala, 1955; Dutton and Greig, 1957). In addition no glucuronides could be isolated from the urine of cats fed various glucuronidogenic substances (Professor R. T. Williams, private communication quoted by Dutton and Greig, 1957.)

Fifty grammes young cat liver were sliced and incubated in 100 c.c. Tyrode buffer containing 0·3 M $MgSO_4$ and 1·25 mg. 8-benzpyrenol with oxygenation.

After extraction a considerable amount of the X_1-type metabolite was obtained on the chromatogram. Once again however, a blue fluorescence was associated with the X_2-type zone. Its ethanol eluate was only weakly fluorescent and its absorption spectrum was more like that of the X_1-type metabolite. Also, unlike the X_2-type, it did not give a coloured ether extract in the naphthoresorcinol test for glucuronic acid.

If the evidence that cats do not produce glucuronides is correct then this result is in accordance with the view that the X_2-type metabolite is the glucuronide. The possibility of the X_1-type being so is eliminated.

The biosynthesis of ^{35}S-labelled X_1-type metabolite

The involvement of inorganic sulphate in the synthesis of ethereal sulphates receives considerable support from the studies of Laidlaw and Young (1953) who observed the excretion of radioactive 2-naphthylsulphuric and 2-amino-1-naphthylsulphuric acids after the simultaneous injection of ^{35}S-labelled inorganic sulphate and 2-naphthol and 2-naphthylamine respectively.

A similar synthesis of ^{35}S-labelled phenylsulphate in liver slices was reported by Sato, Suzuki and Yoshikawa (1953).

In the present experiments 30 g. of mouse liver were sliced and incubated at 37° C. for 3 hours in 150 c.c. Tyrode buffer containing 1·25 mg. 8-benzpyrenol and 0·9 mc. $Na_2\ ^{35}SO_4$ with oxygenation. In a control experiment 8-benzpyrenol was omitted from the mixture.

The slices were then extracted and the distribution of radioactivity determined in the X_1- and X_2-type derivatives and corresponding fractions from the control experiment.

The association of radioactivity with the metabolites was then determined after heating their aqueous solutions under nitrogen in 0·1 N-HCl for 15 minutes, saturating with ammonium sulphate and extracting with ether. Under these conditions 8-benzpyrenol was removed from the X_1-type solution whereas the X_2-type derivative was extracted practically unchanged. The ether extracts were then transferred to 2 c.c. water and their activity determined.

The results of these experiments are shown in Table II.

TABLE II

	Counts per minute	
	Ethanol eluates transferred to 4 c.c. water	Ether extracts of acid hydrolysates transferred to 2 c.c. water
8-Benzpyrenol X_1	1190	67
Control X_1-zone	60	38
8-Benzpyrenol X_2	469	76
Control X_2-zone	328	68

The results indicate a close association of radioactivity with the X_1-type metabolite.

The fact that the activities of the X_2-type zones from the experimental and control runs are of the same order and the fact that this activity is removed under conditions which leave the metabolite unchanged suggests that the activity is due to the presence of ^{35}S-labelled material unconnected with the metabolism of the phenol.

Summary of results

The following facts are considered to establish the identity of the X_2-type metabolite as 8-benzpyrenylglucuronic acid :

(1) Its chromatographic behaviour (cf. Barlow, 1957).
(2) Its relative stability to acid hydrolysis (cf. Bray and Thorpe, 1954).
(3) The colour reaction with naphthoresorcinol reagent.
(4) Its hydrolysis to 8-benzpyrenol by β-glucuronidase and the inhibition of this hydrolysis by 10^{-2} M boiled saccharate solution.
(5) Its synthesis in liver homogenate fortified with crude UDPGA.
(6) The apparent inability of cat liver slices to effect its synthesis.

The following facts are considered to establish the identity of the X_1-type metabolite as 8-benzpyrenylsulphuric acid:

(1) Its chromatographic behaviour (cf. Barlow, 1957).
(2) Its instability to acid (cf. Bray and Thorpe, 1954).
(3) Its hydrolysis to 8-benzpyrenol by takadiastase.
(4) Its synthesis in liver homogenate fortified with ATP and the less consistent results of the " microsome free " supernatant experiments.
(5) The synthesis of ^{35}S-labelled X_1-type metabolite by liver slices in the presence of $Na_2\ ^{35}SO_4$.

Metabolism of the F_1-phenol

The small quantity of the phenol available has severely limited its experimental use so that it has not been possible to confirm the results by repetition.

The general appearance of the opened abdominal cavity was similar to that after 8-benzpyrenol. The intensity of the fluorescence was much reduced by comparison however, presumably due to the much lower dosage level.

Gall bladder, duodenum and small intestine

The combined gall bladder, duodenum and small intestine were extracted as described for 8-benzpyrenol. On chromatographic separation similar blue fluorescent X_1- and X_2-type fractions were obtained.

The ethanol eluate of the X_1-type zone possessed only weak absorption but pronounced maxima were present at 298 and 378–380 mμ with inflections at 362, 369 and 406–410 mμ (Table I). On acidification with hydrochloric acid and gentle warming under nitrogen it was converted back into the F_1-phenol as shown by the appearance of the 418 mμ band in the absorption spectrum and the fluorescent colour change from blue to green in sodium hydroxide.

Strongly absorbing background material prevented possible characterisation of the X_2-type metabolite by absorption spectroscopy but, on transfer to water

and incubation with β-glucuronidase, an ether extract exhibited the characteristic absorption spectrum of the F_1-phenol.

In view of this evidence and by analogy with the behaviour of 8-benzpyrenol it is concluded that the F_1-phenol is similarly conjugated with sulphuric and glucuronic acids.

RE-EXAMINATION OF THE METABOLISM OF 3 : 4-BENZPYRENE AND PYRENE

(a) 3 : 4-*Benzpyrene*

Identification of the X_1-type metabolite of 8-benzpyrenol as the sulphate conjugate is in direct conflict with previous evidence suggesting the presence of a β-glucuronosidic linkage in the X_1 metabolite derived from 3 : 4-benzpyrene. The discovery that 8-benzpyrenylsulphuric acid is instantly decomposed by acid and ether suggested a possible explanation for this discrepancy for in the previous series of experiments, after incubation with β-glucuronidase, the solutions were routinely acidified with hydrochloric acid to facilitate removal of the phenol by ether. It was essential therefore to repeat those experiments under the same conditions used for the 8-benzpyrenyl metabolites.

Effect of acid.—On acidification of the aqueous solution of the metabolites free F_1-phenol was immediately extractable from the BPX_1 solution with ether. The BPX_2 metabolite tended to form a scum at the interface but spectroscopically appeared to be unchanged.

The BPX_2 was then heated in 10 N-HCl at 100° C. under nitrogen for 2 hours. An ether extract of the hydrolysate was yellow in colour with a bright blue fluorescence. It absorption spectrum was predominantly that of the F_1-phenol but attenuation of the 395 and 418 mμ bands suggested the presence of some 8-benzpyrenol. This was verified on transfer to hexane when the 396 and 419–420 mμ bands of 8-benzpyrenol appeared in the F_1 spectrum.

In a further experiment the ether extract of the BPX_2 hydrolysate was transferred to xylene and chromatographed on a column of silica gel over alumina when the phenolic fraction was obtained as a bright yellow/green fluorescent zone on the alumina. The absorption spectrum of the ethanol eluate from this zone was predominantly that of the F_1-phenol but an additional band at 425–426 mμ indicated the presence of 8-benzpyrenol.

Thus, behaviour so far was similar to that of the 8-benzpyrenyl conjugates. A further phenomenon was observed however in the case of the BPX_2 on acid hydrolysis. The xylene filtrate from the silica gel/alumina chromatogram invariaby possessed a blue/violet fluorescence. On evaporating the filtrate to dryness and dissolving the residue in ethanol the characteristic absorption spectrum of 3 : 4-benzpyrene was obtained.

Tests for glucuronic acid.—Solutions of the metabolites were heated with naphthoresorcinol reagent as described for 8-benzpyrenol. Similar colorations were obtained on extraction with ether suggesting the presence of a glucuronide moiety in BPX_2 only.

Effect of β-glucuronidase.—After incubation with β-glucuronidase a trace amount only of the F_1-phenol was removed from the BPX_1 solution by direct extraction with ether. A similar trace was obtained from the boiled enzyme control.

The BPX_2 incubate however, yielded a bright blue fluorescent ether extract

which was not obtained from the boiled enzyme control. The presence of F_1-phenol and a smaller amount of 8-benzpyrenol in the extract was determined by absorption spectroscopy after transfer to hexane.

Effect of takadiastase.—Little blue fluorescent material was extracted from the BPX_2 solution by ether after incubation with takadiastase. The BPX_1 solution however, yielded a bright blue fluorescent extract exhibiting the characteristic absorption spectrum of the F_1-phenol. This contrasted markedly with the weak blue fluorescent extract when the boiled enzyme was used.

Nature of BPX_1 and BPX_2.—The above results invalidate previous conclusions as to the nature of the X_1 metabolite of 3 : 4-benzpyrene but confirm the presence of a β-glucuronosidic linkage in the X_2 metabolite.

In view of this evidence, and by analogy with the behaviour of the 8-benzpyrenyl metabolites, it is concluded that BPX_2 is a complex mixture containing at least three derivatives, the glucuronide of F_1, the glucuronide of 8-benzpyrenol and an acid-decomposable benzpyrene precursor.

The behaviour of BPX_1 is in accordance with it being the sulphate conjugate of F_1 only as 8-benzpyrenol has not, so far, been identified by absorption spectroscopy as a product of its hydrolysis. The possibility of a small amount of 8-benzpyrenol being present in the F_1 cannot entirely be ruled out however, for in a parallel case, Conney, Miller and Miller (1957) have reported that a methylated metabolite possessing the characteristic absorption spectrum of 8-methoxybenzpyrene did in fact contain some of the 10-methoxyderivative.

Attempted fractionation of BPX_1 by partition chromatography has failed to establish the presence of 8-benzpyrenyl-sulphuric acid. Such a result is not surprising perhaps in view of the very similar properties of, and the close spectral relationship between, the metabolites and the fact that only a small amount of free 8-benzpyrenol appears in the caecum and large intestine during the first few hours following intravenous injection of 3 : 4-benzpyrene.

It seemed possible that more direct evidence for its presence would be afforded by an investigation of the BPX_1 obtained after the intraperitoneal injection of the hydrocarbon for, under these conditions, the amount of 8-benzpyrenol initially appearing in the faeces is increased (Weigert and Mottram, 1946). Accordingly the X_1 metabolite was isolated from the small intestines of mice killed 6 hours after the intraperitoneal injection of 1 mg. of 3 : 4-benzpyrene. There was no marked difference in its absorption spectrum but, on mild acid hydrolysis, a mixture of F_1 and 8-benzpyrenol was obtained as shown by the presence of doublets in the absorption spectrum at 392 and 396 mμ and 415 and 419–420 mμ in hexane as solvent (Harper, 1958*b*).

This fact therefore is considered to provide good evidence for the metabolic formation of 8-benzpyrenylsulphuric acid from 3 : 4-benzpyrene.

Attempts to fractionate the BPX_2 complex were equally unsuccessful in that separation into the respective glucuronides was not achieved. It was established however, that the hydrocarbon precursor is concentrated in a narrow tan coloured surface zone of the chromatogram and its presence appears to be associated with the appearance of a 346 mμ maximum in the BPX_2 absorption spectrum (Fig. 3). Successive lower fractions of the zone possessed similar absorption spectra but slight variations in the position of the absorption maxima were noted. The significance of these shifts has not been established but it is suggestive of the presence of more than one metabolite.

The absorption spectrum of BPX_2 obtained in the filtrate after development of the chromatogram with ethanol is also recorded here (Fig. 3) as it is considered to establish the fully aromatic benzpyrenoid configuration of the metabolite.

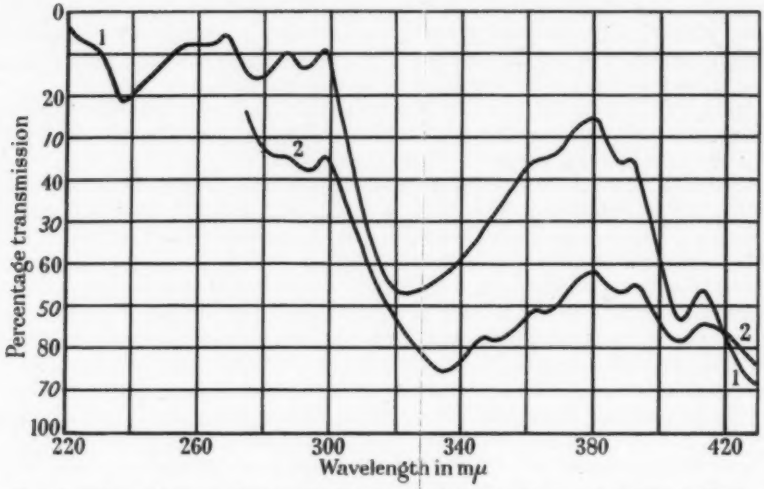

FIG. 3.—Absorption spectra in ethyl alcohol. BPX_2 fractions: 1. Ethanol filtrate. 2. Top surface zone. (For explanation see text.)

(b) *Pyrene*

In a previous study of the metabolism of pyrene two intermediate metabolites were isolated (Harper, 1958*a*). One was identified as 3-pyrenylglucuronic acid and appeared to be the major metabolite excreted in the bile. The other, signified as PX, was only obtained from the *in vivo* liver but was formed, together with the glucuronide and free phenol, from pyrene in isolated liver slices. It was rapidly decomposed to 3-hydroxypyrene by acid and in view of the similar behaviour found for 8-benzpyrenylsulphuric acid the possibility of it being 3-pyrenylsulphuric acid has been tested.

Separation of the pyrene metabolites in the small intestine into X_1 and X_2-type fractions was carried out as described for 8-benzpyrenol.

The X_2 zone yielded a blue/violet fluorescent solution exhibiting the characteristic absorption spectrum of 3-pyrenylglucuronide. This was confirmed by its hydrolysis to 3-hydroxypyrene both by β-glucuronidase and acid. The presence of a small amount of free pyrene in the acid hydrolysate was also established by xylene extraction followed by chromatography on silica gel over alumina. This is in accordance with the presence of an acid-labile precursor of pyrene previously reported in the faeces (Harper, 1957).

The X_1 zone yielded a pale blue fluorescent solution exhibiting the characteristic absorption spectrum of PX. The PX remained unaffected on incubation with β-glucuronidase but was rapidly hydrolysed to 3-hydroxypyrene by takadiastase.

It is concluded therefore that PX is 3-pyrenylsulphuric acid and that, in like

manner to 8-benzpyrenol, 3-hydroxypyrene is eliminated in the bile in conjugation with both glucuronic and sulphuric acids.

OTHER HYDROCARBONS

In the case of two other hydrocarbons, chrysene and 20-methylcholanthrene, the same pattern of biliary excretion has been observed. X_1- and X_2-type derivatives have been isolated from the duodenum and small intestine and these have undergone hydrolysis to phenolic compounds in the caecum and large intestine.

An acid-decomposable precursor of 20-methylcholanthrene has also been found in its X_2 complex.

DISCUSSION

Although the absolute identification of the metabolites of 3 : 4-benzpyrene must eventually lie in their individual isolation and characterisation, with chemical synthesis as the final check, the results of these experiments are considered to provide good evidence for the presence of sulphate and glucuronide conjugates of the F_1-phenol and 8-benzpyrenol in the mixture of metabolites excreted via the gall bladder. The formation of such compounds was of course to be anticipated from the metabolic studies carried out by Conney, Miller and Miller (1957). These workers investigated the metabolism of 3 : 4-benzpyrene in fortified liver homogenate and identified 8-benzpyrenol, 10-benzpyrenol and the F_1-phenol as major products of oxidation. As 10-benzpyrenol has also been identified as an excretion product of 3 : 4-benzpyrene in the faeces (Berenblum and Schoental, 1946) a similar sequence of conjugation and hydrolysis is to be anticipated but the present experiments have yielded no evidence on this point. In this connection it is perhaps significant that Chalmers (1956), using more refined paper partition chromatographic methods, has isolated a metabolic fraction possessing an absorption spectrum suggestive of 10-methoxybenzpyrene and giving a positive test for glucuronic acid.

The fact that the F_1-phenol has not been detected as an excretion product of 8-benzpyrenol in these experiments suggests that it is formed as a separate entity during the primary oxidation of 3 : 4-benzpyrene in the liver. The findings of Conney, Miller and Miller (1957) previously referred to are consistent with this view. If this is the case then a possible metabolic sequence would be as in Fig. 4.

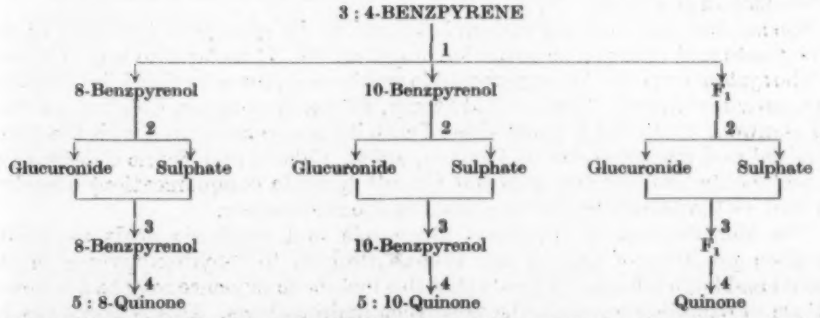

FIG. 4.—1. Oxidation by microsome/TPNH system of tissue. 2. Conjugation with glucuronic and sulphuric acids. 3. Hydrolysis in caecum and large intestine. 4. Partial oxidation to quinones.

The unknown factors in this scheme are the identity of the F_1-phenol and the mechanism by which the sulphate conjugates are hydrolysed in the gut. No new evidence has been forthcoming on the former point but the acid-labile nature of the sulphate conjugates suggests that possible pH changes down the gut could play a part in their hydrolysis, as suggested by Weigert and Mottram (1946). The other possibility is hydrolysis by arylsulphatase activity, and on this point no conclusive information appears to exist. Dodgson, Spencer and Thomas (1953) reported that the intestine possessed but little arylsulphatase activity but this result was based on the activity of only small pieces of the intestine, the exact location of which was not stated. Indeed, in view of the fact that a spectrophotometric method of assay was used, it is unlikely that the highly coloured caecal contents would be amenable to such an investigation.

Obviously further work must be done to establish this point but it is perhaps significant that certain strains of bacteria have been found to possess arylsulphatase activity (e.g. Whitehead, Wildy and Engbaek, 1953).

The failure of Gutmann and Wood (1950) to obtain evidence of ^{35}S-labelled metabolites of 3 : 4-benzpyrene from the urine of animals previously dosed with ^{35}S-labelled L-cystine and DL-methionine appears to be at variance with the biosysnthesis of radioactive 8-benzpyrenylsulphuric acid reported here. It must be borne in mind however that only a relatively small percentage (17 per cent according to Heidelberger and Weiss, 1951) of injected 3 : 4-benzpyrene is eliminated in the urine. The present experiments suggest that only a small fraction of this can be attributed to the presence of the sulphate conjugates and of course further hydrolysis to the phenols presumably takes place during the collection period. It is doubtful therefore whether such a small increase in activity would be detected in an experiment where an appreciable standard deviation existed. In the same experiment the failure to obtain evidence of increased excretion of radioactivity in the faeces can be explained by resorption of the sulphate radicals after hydrolysis of the conjugates in the caecum. That the colon is an active site of sulphate fixation is shown by the work of Pasternak, Kent and Davies (1958), and Pasternak and Kent (1958).

The identification of the X_1 and X_2 metabolites of 3 : 4-benzpyrene as sulphate and glucuronide conjugates respectively can now be correlated with the findings of Calcutt and Payne (1954) with respect to the intracellular distribution of the metabolites in the liver.

The sulphate synthesising system is located in the supernatant fraction of the homogenate and it was in this fraction only that the X_1 metabolite was detected.

The system responsible for glucuronide synthesis on the other hand, is located in the microsomal fraction (Dutton and Storey, 1954 ; Strominger, Kalckar, Axelrod and Maxwell, 1954), but a lower level of activity is also associated with the mitochondrial and nuclear fractions (Dutton, 1956). Calcutt and Payne obtained the X_2 metabolite from all fractions but Calcutt (private communication) considers the bulk of the metabolite to be located in the microsomes.

The identification of 3-pyrenyl glucuronic and sulphuric acids as biliary excretion products of pyrene and their hydrolysis to 3-hydroxypyrene in the caecum and large intestine suggests that this metabolic sequence may be a common pattern of excretion amongst the polycyclic hydrocarbons. This is borne out by the isolation of X_1- and X_2-type derivatives of chrysene and 20-methylcholanthrene. Chalmers (1957) has also reported the excretion of conjugated metabolites of

anthracene in the bile. The possibility of a similar sequence for 1 : 2 : 5 : 6-dibenzanthracene is suggested by the unchanged excretion in the faeces of the unidentified phenolic metabolite from the rabbit after its reinjection into mice (Dobriner, Rhoads and Lavin, 1942).

The formation of acid-labile precursors of the hydrocarbons may also be a common factor. The excretion of such compounds has already been reported for a variety of hydrocarbons (Chang and Young, 1943), and a similar behaviour has now been observed for pyrene, 3 : 4-benzpyrene and 20-methylcholanthrene.

SUMMARY

1. The excretions of 8-benzpyrenol and the F_1 metabolite of 3 : 4-benzpyrene have been investigated after intravenous injection in the mouse. The same pattern of excretion has been observed as that arising from injection of the parent hydrocarbon i.e., the elimination of X-type derivatives in the bile and their hydrolysis back to the phenols in the caecum and large intestine.

2. The X_1- and X_2-type metabolites of 8-benzpyrenol have been identified as the sulphate and glucuronide conjugates of the phenol respectively and similar structures are proposed for the metabolites derived from the F_1-phenol.

3. A re-examination of the metabolism of 3 : 4-benzpyrene has invalidated previous conclusions as to the nature of the X_1 metabolite (BPX_1) but has confirmed the presence of glucuronic acid in the X_2 metabolite (BPX_2).

4. Evidence has been obtained that BPX_2 is in fact a complex mixture containing the glucuronides of F_1 and 8-benzpyrenol together with an acid-labile precursor of 3 : 4-benzpyrene.

5. The behaviour of BPX_1 is in accordance with it being the sulphate conjugate of F_1 only but evidence for the presence of 8-benzpyrenylsulphuric acid in the BPX_1 has been obtained after the intraperitoneal injection of the hydrocarbon.

6. A possible metabolic sequence to account for these facts is proposed.

7. A re-examination of the metabolism of pyrene has been made. The hitherto unidentified metabolite isolated from the liver has now been identified as 3-pyrenylsulphuric acid and found to be an excretion product in the bile together with 3-pyrenylglucuronic acid and an acid-labile precursor of the hydrocarbon.

8. Chrysene and 20-methylcholanthrene have yielded similar X_1- and X_2-type metabolites undergoing hydrolysis to phenolic products in the caecum. It is suggested that this may be a common pattern of excretion for the polycyclic hydrocarbons.

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VARIATION OF β-GLUCURONIDASE LEVELS WITHIN THE C_3H STRAIN AND OBSERVATIONS ON THE EFFECT OF POLYCYCLIC HYDROCARBONS ON GLUCURONIDASE ACTIVITY

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β-GLUCURONIDASE is present in most animal tissues and is often found in highest amount in organs exposed to oestrogen stimulation and in malignant tumours. The main action of the enzyme is not accurately defined but Fishman (1955) has summarized the evidence in favour of its importance in steroid metabolism and participation in glucuronide synthesis. Levvy (1953) and co-workers proposed that β-glucuronidase levels represented an index of tissue growth and proliferation. However a number of examples in which there is no such correlation have been reported, e.g. by Mills, Paul and Smith (1953). The work described in this paper arose out of attempts to correlate glucuronidase levels with tumour production and growth.

Fishman and Farmelant (1953) described the action of androgens and oestrogens on liver and kidney β-glucuronidase in inbred strains of mice. They found certain characteristic responses to these hormones, namely that oestrogen (stilboestrol) produced an increase in the β-glucuronidase level of liver, while the kidney was not affected. Conversely, androgens (testosterone propionate) produced increases in the enzyme level in the kidney and did not change the activity in the liver. These observations were generally consistent for male mice of the strains tested. It seemed that this difference in response might offer a biochemical method of testing compounds for possible oestrogen or androgen-like action. The possibility of a stimulating action by glucuronidogenic substances would, of course, have to be borne in mind.

On this basis the effects of injections of a number of polycyclic hydrocarbons into mice have been examined. The main point of interest was to seek an explanation of the remarkable effect of 9 : 10-dimethyl-1 : 2-benzanthracene in producing high yields of ovarian and breast tumours, particularly in IF strain mice (Howell, Marchant and Orr, 1954). These results are presented in the later part of the paper.

The level of β-glucuronidase activity in certain tissues at least, appears to be under genic control. Morrow, Greenspan and Carroll (1949) first observed that the inbred strain C_3H, possessed a much lower level of liver β-glucuronidase (about one-tenth) than was found in other strains. The levels in kidney and mammary tissues were also surprisingly low. Subsequently another "low" strain was discovered, Bar Harbor-AKR (Fishman and Farmelant, 1953). By cross-mating mice of strains C_3H (low activity) and strain A (high), Law, Morrow and Greenspan (1952), were able to demonstrate that the enzyme level was determined genetically and that the higher glucuronidase activity of strain A appeared to be associated with a dominant gene. The low C_3H strain was presumed to carry the corresponding recessive alleles. At the commencement of the work

described here an examination was made of the liver and kidney β-glucuronidase activities of the inbred strains maintained in the Cancer Research Laboratories, University of Birmingham. It was then discovered that of two C_3H substrains from different sources, one had "high" and the other has "low" enzyme activity. The derivation of these substrains was examined and an attempt made to detect the origin of this divergence in a supposedly uniform inbred strain. This investigation forms the main part of the work reported here and is described first. A preliminary account has been given of some aspects of this work (Hamer, 1955).

METHODS

Animals.—Inbred mice maintained by brother-sister mating in the Birmingham laboratories were used, except where separate origin is indicated. All mice were housed in the same conditions and had water and a cube diet available throughout.

Enzyme estimation.—Homogenates of the tissue being studied were prepared using a few strokes by hand in a glass homogenizer. About 100 mg. of tissue were taken, rapidly weighed and then suspended in fifty times its amount of 0·1 M acetate buffer, pH 4·5. The activity of the enzyme, β-glucuronidase, in this homogenate was determined using phenolphthalein glucuronide as a chromogenic substrate, the phenolphthalein liberated being determined colorimetrically. The substrate, phenolphthalein-β-D-glucuronide was prepared by the method of Fishman, Springer and Brunetti (1948) and the estimations were carried out as described by Fishman (1950). After a suitable hydrolysis time the phenolphthalein produced was measured in a glycine buffer of pH 10·45 using an "EEL" photoelectric absorptiometer and filter 605. All estimations were done in duplicate. Activity is expressed as μg. phenolphthalein liberated per hour g. of tissue taken at 37° C. under the conditions of the estimation.

Treated mice.—A number of experiments are described in which mice received injections of hormones or hydrocarbons. Injections of these compounds were given intraperitoneally in arachis oil solution, 5 mg./ml. 0·1 ml. was injected on alternate days for two weeks, representing a total dosage of 3·5 mg. compound.

RESULTS

As a preliminary investigation a survey was made of the strains available in the Birmingham laboratories and some of these results are given in Table I. The activities found are somewhat lower than those recorded by the American workers but the general pattern is the same.

TABLE I.—*β-glucuronidase Activity in Male Mice of Various Strains*

Strain	Activity in glucuronidase units	
	Liver	Kidney
A	3210	2100
C57Bl	2950	1535
IF	3170	2400
C_3H (ex Pollards Wood)	3150	1590
C_3H (ex Leeds)	270	370
Outbred Albino	3390	1370

(Activities expressed as μg. phenolphthalein liberated per hour per g. wet. tissue)

All strains showed comparable levels except for a group of C_3H mice which were found to have much lower liver and kidney glucuronidase levels, as had been expected from the earlier work referred to. However one surprising finding was that another group of C_3H mice of different origin showed the higher level of activity. This was surprising as the two substrains had been maintained under similar conditions for about five years. One, the high strain, had been obtained from C_3H mice originally supplied by Pollards Wood Research Laboratories of the Chester Beatty Institute (Dr. Carr, Dr. Foulds). The "low" strain was developed from mice supplied by Department of Cancer Research, University of Leeds (Dr. Dmochowski, Dr. Bonser). This pronounced difference within an inbred strain was surprising and so a survey was carried out of some of the C_3H colonies available at different centres, and which were known to have a common origin. The results obtained in liver and kidney glucuronidase assays on these mice are listed in Table II.

TABLE II.—*β-glucuronidase Activity in Male Mice from Various C_3H Substrains*

Substrain origin	Glucuronidase activity: Liver	Glucuronidase activity: Kidney	Separation date	General type
Birmingham (ex Pollards Wood)	3150	1590	1950	High.
Pollards Wood	2520	1440	Parent line	,,
Glasgow	2910	2160	1948	,,
Birmingham (ex Leeds)	270	370	1941	Low.
Imperial Cancer Fund	224	1300†	1941	,,
University College, London (ex C_3H/Bi)*	248	427	Pre-1938	,,
University College, London (ex C_3H/St)*	218	382	Pre-1938	,,

* These were substrains maintained at University College, London and were descendants of mice separated in U.S.A. prior to the establishment of the strain in Britain in 1938 (see McLaren and Michie, 1954).

† This strain was the only case in which any pronounced sex difference was found. The corresponding values for female mice were: liver 252 units, kidney 200. The high activity in male kidney will be discussed later.

Fig. 1 shows the origin of these substrains and their interrelation. All were derived from an original stock obtained from Bittner (Bar Harbor) in 1938. This data is the relevant part of a more complete survey of C_3H colonies prepared by McLaren and Michie (1954).

The substrains tested fall distinctly into groups of "high" (2500–3000 units) or "low" (200–300 units) liver β-glucuronidase activity. In all but one case, which will be discussed later, the kidney levels follow the same pattern. Two substrains obtained from the United States fairly recently and two British substrains separated before 1941 (Imperial Cancer Research Fund and Leeds), had low levels of glucuronidase activity. However two groups separated after 1948 and the descendants of the original colony, all gave high values. These results would suggest that some change took place in the breeding stock now maintained at the Pollards Wood Laboratories, during the period 1941 to 1948. Whatever the nature of this change was, it finds expression as a difference in the β-glucuronidase activity in the liver, kidney and presumably other tissues, e.g. mammary tissue (Cohen and Bittner, 1951).

By cross-breeding a strain of "high" activity (A) with one of low activity (C_3H), Law, Morrow and Greenspan (1952) showed that the presence of high

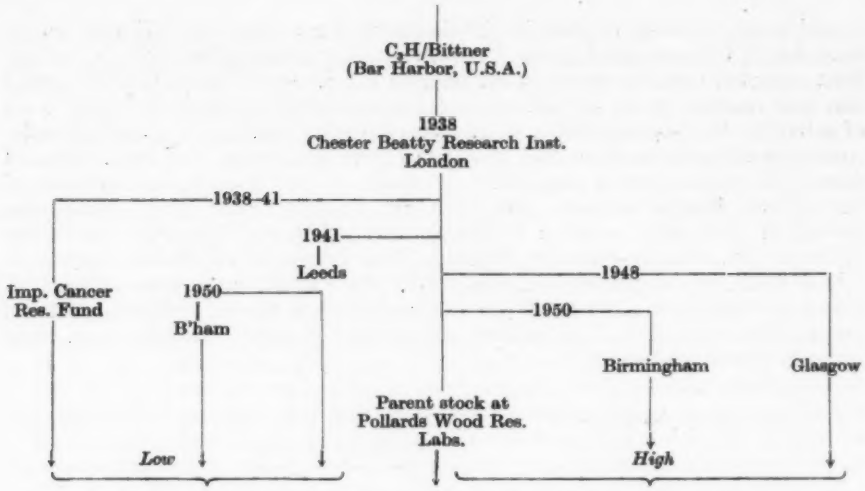

FIG. 1.—The origin of the various C_3H colonies examined (cf. Table II).

glucuronidase activity appeared to be associated with a dominant gene. A series of measurements have been carried out on hybrid mice produced by crossing C57 (high) with C_3H (low). The results are summarised in Table III.

TABLE III.—*β-glucuronidase Activity in Mice Obtained by Crossing "High" and "Low" Strains*

Animals		Glucuronidase activity: Liver	Glucuronidase activity: Kidney
C_3H ♀ × C57 ♂	Males	1940	2020
	Females	2050	960
C57♀ × C_3H ♂	Males	1840	1130
	Females	2080	1080
C_3H (Imp. Cancer Res. Fund)	Males	224	1300†
	Females	252	200
C57	Males	2950	1535
	Females	3060	1400

† See relevant footnote in Table II.

The results tend to confirm those of Law *et al.* referred to. The enzyme activities in the hybrid mice are not quite so high as those in C57 mice but are nevertheless about eight times greater than those of the C_3H strain. There was no evidence of any significant maternal effect though such an effect had been observed in the case of another intra-strain variation, namely in the number of lumbar vertebrae in mice (McLaren and Michie, 1955).

Effect of hydrocarbon injections on β-glucuronidase levels

The hydrocarbon of particular interest to this investigation was 9 : 10-dimethyl-1 : 2-benzanthracene which had been found to produce many ovarian and breast

tumours. It was hoped that some evidence of a hormone-like action might be found. Since many compounds such as phenols, etc., give rise to increases in glucuronidase activity, two closely related hydrocarbons were tested for comparison. These were 1 : 2-benzanthracene and 1 : 2 : 5 : 6-dibenzanthracene. The results obtained with male mice of two strains are given in Table IV.

TABLE IV.—*Effect of Hydrocarbon Injections on Liver and Kidney Glucuronidase Levels*

Treatment	A Strain		IF Strain	
	Liver	Kidney	Liver	Kidney
Control (oil only)	2800	2210	3360	2030
1 : 2-benzanthracene	2980	2050	3840	1770
1 : 2 : 5 : 6-dibenzanthracene	3020	2150	4050	1890
9 : 10-dimethyl 1 : 2-benzanthracene	3400	2090	4330	1400
Stilboestrol	4860	1920	4825	2090

Dosage : 3·5 mg. in oil spread over 14 days. Results are average of ten determinations in male mice.

It will be seen that all hydrocarbons produced an increase in the liver glucuronidase activity. In the case of 9 : 10-dimethyl-1 : 2-benzanthracene, this increase was about one-third to one-half that produced by injection of an equivalent weight of stilboestrol. No increase occurred in the level of glucuronidase in the kidney, indeed with IF strain mice there was an actual decrease. On the basis of the results of Fishman and Farmelant (1953) it might therefore be supposed that 9 : 10-dimethyl-1 : 2-benzanthracene was having an oestrogen-like action on the enzyme level. It did not however appear safe to make this deduction as it was found that the injections of this hydrocarbon could produce a considerable toxic reaction. Of several strains tested, only IF and A mice seemed able to survive the period of the experiment at the dosage given. The usual effect of the injections was to cause the development of considerable amounts of ascitic fluid by about the tenth day of the total period of fourteen days. This must presumably represent some toxic action on the liver and kidneys. It does not appear safe therefore until further investigations have been made to compare the action of this hydrocarbon on the enzyme with that of stilboestrol. 1 : 2 : 5 : 6-dibenzanthracene occasionally produced small amounts of ascites but none was formed by arachis oil alone, 1 : 2-benzanthracene or stilboestrol. However, it was observed that the production of an inflammatory reaction by intraperitoneal injection of kieselguhr had no effect on the glucuronidase levels.

DISCUSSION

The results presented of investigations of various C_3H colonies, demonstrate the occurrence of a striking variation in the case of the level of activity of the enzyme β-glucuronidase. Only an approximate dating of this change is possible, namely sometime between 1941 and 1948. During this period the parent colony was moved from London to Edinburgh, and then later back to the Pollards Wood Research Laboratories of the Chester Beatty Research Institute, London. Whether the change was in fact a mutation and then a chance selection of " high " mice for

breeding, cannot be determined. There are, in the literature, examples of related variations. Morrow, Greenspan and Carroll (1950) surveyed a number of strains and reported that out of 80 C_3H (Heston) mice examined, three mice (all males) showed a high level of activity of the liver glucuronidase. The same workers also recorded a difference between two CBA sublines. CBA (Andervont) were low while CBA (Strong) mice were of the high group, having liver glucuronidase activity about fifteen times the former. No history of the divergence was presented but Greenstein (1954) commenting on this intra-strain divergence raises the question as to whether, because of this difference, the two types of CBA mice should rather be looked upon as entirely new strains.

It is not known whether the difference in enzyme level in two branches of the C_3H strain is associated with any difference in tumour incidence. Insufficient animals were available for a quantitative comparison but from general observation the incidence of mammary tumours appeared to be about the same in the " high " and " low " Birmingham substrains. One interesting feature was encountered in the case of the C_3H colony maintained at the Imperial Cancer Research Fund, London (Dr. Craigie). As recorded in Table II, male mice of this colony showed an exceptionally high glucuronidase level in the kidney (1300 units) as compared with the typical " low " level in the liver (224 units) and low values in female liver and kidney (252 and 200 units, respectively). The range of male kidney values was wide, from 600 to 2000 units, and may have varied with age although this was not examined experimentally. However the notable feature of this substrain is that the male mice develop a high incidence of spontaneous benign hepatomas (Craigie, 1954, 1955). These hepatomas are reported to appear from seven months onwards and by twelve months the incidence was 70 per cent. Only five hepatomas were found in 90 females, all over twelve months old. So in this one case there seemed to be two striking divergences in the male mice, (*a*) abnormally high-glucuronidase activity in the kidney and (*b*) early and high incidence of spontaneous hepatomas. Only further investigation can show how closely these two factors are related. In the cross breeding experiments presented in Table III between C_3H mice of the Imperial Cancer Fund substrain and C57 mice, it will will be seen that the male offspring of the C_3H ♀ × C57 ♂ cross have a much higher kidney glucuronidase activity than the other groups. This rather suggests that the " high " kidney enzyme factor is passed on through the C_3H female to the males of the next generation. However in preliminary experiments with other cross-matings this could not be confirmed.

To date the " high " or " low " chracteristic of inbred mouse strains of mice has been the only considerable biochemical strain difference reported (Russell, 1955). However the difference can hardly be an abnormality as, say, phenylketonuria in man, since the mice are comparable in health, weight, etc., in both high and low strains. Only in the one group discussed above does there seem to be a possible association of an abnormal glucuronidase level with a tumour incidence—and there is no evidence that this particular variation is a genetic one. So far as other intra-strain variations are concerned, a number of other variations have been observed (Gruneberg, 1954). McLaren and Michie (1954) have examined a large number of C_3H substrains and have found certain genetically controlled skeletal variations. One group of C_3H substrains has predominantly five, the other has six, lumbar vertebrae. The C_3H substrains examined here all fall into the group having five lumbar vertebrae. Also all the substrains were

identical in coat colour. Obviously any variations of the type reported here must be considered carefully, particularly when comparing results involving for example hormone mechanisms, obtained in different laboratories.

As already pointed out in the preceding section, attempts to use the characteristic response of the liver and kidney glucuronidase levels to detect oestrogen and androgen influences, were inconclusive in the case of the hydrocarbons studied. The toxic reactions produced by the administration of 9 : 10-dimethyl-1 : 2-benzanthracene may, or may not, have been associated with the accompanying increase in the liver glucuronidase level.

SUMMARY

1. Differences were found between various colonies of C_3H mice. Some of these had a high level of β-glucuronidase activity in the liver and kidney (2500–3000 units while others had much lower levels (200–300 units). Earlier workers had shown that these characteristic enzyme levels represented a genetically controlled difference and so an attempt was made to trace the origin of the divergence in the colonies.

2. Only in one colony of mice examined was there a possible correlation between enzyme activity and tumour incidence. In one substrain, male mice having an abnormally high glucuronidase activity in the kidney, were also found to have an early and high incidence of hepatomas.

3. Liver and kidney glucuronidase levels respond differently to androgens and oestrogens. Mice were given injections of polycyclic hydrocarbons in solution and it was found that 9 : 10-dimethyl-1 : 2-benzanthracene produced a similar response to stilboestrol but of about one-third the strength. The results were however, complicated by the toxicity of the hydrocarbon, making direct comparisons of the enzyme response unsure.

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MYELOPEROXIDASE ACTIVITY IN BONE MARROW EXTRACTS FROM TUMOR-BEARING MICE

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THE host response to neoplasia may be quite variable depending on the parameter observed, the strain or species of animal studied, nutritional state of the animal, and other factors. Of the various parameters considered, the one which stands out as the most nearly universal host response to early neoplasia is the depression in hepatic catalase activity. This subject has been reviewed by Greenstein (1954). The enzyme delta-aminolevulinic acid dehydrase may show a response similar to that of catalase (Tschudy and Collins, 1957). The underlying mechanism of this catalase response has been studied extensively and has led to a consideration of the following factors: (1) That the tumor elaborates a "toxohormone" which depresses catalase activity (Nakahara and Fukuoka, 1948; Fukuoka and Nakahara, 1951; Greenfield and Meister, 1951; and Ceriotti and Spandrio, 1955); (2) that the depression may be related to the presence of excess sulphydryl groups or ascorbic acid which could give rise to an inactive catalase-H_2O_2 complex (Alexander, 1957); and (3) that the effect of a neoplastic growth may be similar to that produced by administration of 3-amino-1,2,4-triazole (Heim, Appleman and Pyfrom, 1955; Heim, Appleman and Pyfrom, 1956; Sugimura, 1956; and Feinstein, 1958). This question is still unresolved.

Since the depressed hepatic catalase activity in tumor-bearing animals has been established beyond doubt, it seemed worth while to investigate an enzyme system whose function might be related in some way to the level of catalase activity. With this in mind we undertook the study of myeloperoxidase (MPO) in tumor-bearing mice. Myeloperoxidase, formerly called verdoperoxidase and studied in detail by Agner (1941), is an iron-porphyrin- containing enzyme which utilizes hydrogen peroxide as a substrate, the enzyme-substrate complex then having the capacity to oxidize donor molecules such as aromatic amines, phenols, and natural compounds such as epinephrine, ascorbic acid, etc. (Theorell, 1951). Since this enzyme has thus far been demonstrated only in cells of the myeloid series, this study was carried out on bone marrow tissue.

MATERIALS AND METHODS

In order to develop quantitative data on a tissue such as mouse bone marrow, we adopted the procedure of homogenizing the whole, cleaned mouse femur in a Virtis "23" homogenizer. The femur was scraped clean of all adherent tissue so as not to vitiate subsequent protein determinations performed on the extracts. Some control observations on bone marrow tissue taken from rat femur gave figures of the same order of magnitude as that obtained on the whole homo-

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In order to develop quantitative data on a tissue such as mouse bone marrow, we adopted the procedure of homogenizing the whole, cleaned mouse femur in a Virtis "23" homogenizer. The femur was scraped clean of all adherent tissue so as not to vitiate subsequent protein determinations performed on the extracts. Some control observations on bone marrow tissue taken from rat femur gave figures of the same order of magnitude as that obtained on the whole homo-

genized femur, so it was assumed that components of the bony matrix did not significantly alter the activity of myeloperoxidase (MPO).

As reported by Bancroft and Elliott (1934), and earlier workers, we found that extraction of tissue with phosphate buffer at pH 7·0 removed approximately 50 per cent of the total MPO activity which could be detected. Following a procedure suggested by Wattiaux and de Duve (1956), and explored also by Adams and Burgess (1957), we tried tissue extractions with phosphate buffer containing 0·1 per cent Triton X100 and obtained activity levels substantially greater than that obtained without Triton, approaching 100 per cent of the detectable enzyme activity. A word of caution should be mentioned at this point. In some extracts subjected to freezing at — 70° C., and thawing, additional MPO activity could be detected. It is presumed that some finely divided material was present in the extract and that this underwent structural alteration liberating the additional activity (see below). We have been unable to elicit this effect consistently. Although our standard procedure now allows the low temperature storage of tissue, but not the extract, and involves treatment of the homogenate with *n*-butanol followed by assay of the extract within a few hours, it was considered desirable to record some of our observations on stored extracts, prepared with and without *n*-butanol. These observations are discussed below.

Since phosphate buffer of low ionic strength is generally used in the assay reaction mixture, and phosphates have been reported to weaken the peroxidase reaction with guaiacol (Ostrovskaja, 1950), it seemed advisable to select a different buffer, and one which would be less influenced by the composition of the sample. For these reasons tris buffer at pH 7·0 was tested and found to give results comparable with phosphate buffer.

Increasing the ionic strength of the guaiacol reagent with sodium chloride increased the measured activity. With a final sodium chloride concentration in the reagent of 0·85 per cent, the activity was 34 per cent greater than at 0·45 per cent. With 1·25 per cent NaCl concentration, the activity was only 10 per cent greater than at 0·85 per cent.

Treatment of the homogenate with *n*-butanol for 5 minutes served to break the emulsion which formed during homogenization, and also increased the activity by 5–10 per cent. It was found, however, that when extracts were prepared with *n*-butanol and stored at — 70° C. protein denaturation occurred more rapidly than when *n*-butanol was not used. As noted below, extracts prepared with *n*-butanol and stored at — 70° C. seemed to liberate more activity, if not stored too long, and to suffer more rapid denaturation when storage was prolonged.

Reagents

Stock tris solution : 0·2 M tris-(hydroxymethyl)-aminomethane (Sigma 7–9). Tris buffer, 0·02 M : 100 ml. of 0·2 M tris and 94 ml. of 0·2 N HCl made to 1 L, pH = 7·0–7·1. Stock H_2O_2, 1·0 M : made from 30 per cent H_2O_2 and standardized with permanganate. Working solution H_2O_2, 0·1 M : 1 : 10 dilution of stock solution, prepared fresh each week. Tris-triton solution : 1 : 1 mixture of 0·02 M tris buffer and 0·2 per cent Triton X100 (Rohm and Haas Co.). Sodium chloride solution : 1·70 per cent NaCl. Guaiacol reagent : 0·22 g. guaiacol, cryst. (Amend), made to 100 ml. with a 1 : 1 mixture of 0·02 M tris buffer and 1·70 per cent NaCl, prepared fresh each week.

Procedure

The mouse is sacrificed with ether, the hind limbs are promptly disarticulated and the femurs removed and stored at — 15° C. for at least 10 minutes. The frozen femurs are then carefully trimmed and scraped free of all adherent tissue, transferred to small test tubes and stored at — 70° C. until taken for assay. In control studies no significant differences in MPO activity were observed in femurs stored up to 17 days. Longer storage periods were not tested. The frozen femur is cut with scissors into the stainless steel Virtis homogenizing vessel which contains 0·5 ml. of tris-Triton, and is kept cold with ice cubes. Homogenization is carried out at 13,000 r.p.m. for 2 minutes. While the shaft is revolving slowly, 25 μl. of *n*-butanol (5 per cent) are added gradually. The emulsion is readily broken and stirring is continued for 5 minutes. The cold homogenate is transferred to a cold 1 ml. microcentrifuge tube, the steel vessel is rinsed with 0·3 ml. and 0·2 ml. of tris-Triton solution, and the rinsings are added to the homogenate and mixed. The tubes are then centrifuged in the cold (4° C.) for 3 minutes at 10,000 r.p.m. (5200 G.). The supernatant fluid is then decanted and kept in an ice-bath until assayed. The assay is performed at three levels (25–100 μl.) essentially as described by Colowick and Kaplan (1955), using k_1 conditions. Since $k_1 = \frac{0{\cdot}22}{e\,\Delta t}$ we have expressed our activity units as "e" $= ek_1 = \frac{0{\cdot}22}{\Delta t}$ where e is the enzyme concentration in M/L and Δt is the time, in seconds, for an increase in optical density of 0·05, after addition of hydrogen peroxide. When the k_1 dissociation constant is accurately known (of the order of 10^7), "e" can readily be translated into absolute units.

Protein determinations on the bone marrow extracts were performed with the biuret procedure of Gornall, Bardawill and David (1949), using Armour's crystalline bovine albumin as a standard. Specific activity values are expressed as "e" per gram of protein.

Experimental tumors

Bone marrow MPO levels were measured against time after inoculation or implantation with three different types of tumor, the Ehrlich ascites carcinoma, C-1498 myelogenous leukaemia, and Sarcoma 180. Twenty-gram mice were employed in all cases, using the C57 Bl mice for the C-1498 implants and Swiss Webster mice for the other two tumors. The Ehrlich ascites carcinoma was inoculated, intraperitoneally, as 0·1 ml. of a 7-day growth. This inoculum was assumed to contain 10^7 cells. The C-1498 and S-180 tumors were implanted as tumor bits in the right axillary region with the conventional trocar technique. Control mice for the Ehrlich ascites experiments received intraperitoneal injections of normal saline; controls in the C-1498 experiment were untreated; and controls in the S-180 experiment were subjected to sham implants by trocar.

RESULTS

Ehrlich ascites carcinoma

In a preliminary experiment involving three control mice and three inoculated with Ehrlich ascites carcinoma, one experimental animal and its control were sacrificed and assayed for MPO on days 2, 6, and 8 after inoculation. Extracts

of the left and right femurs of each animal were prepared without *n*-butanol and assayed separately without storage. The MPO specific activity showed a linear increase against time amounting to 7·7 per cent per day. The same experiment was repeated using four control and six experimental mice (Expt. EMA-I). The latter were sacrificed in groups of two on days, 1, 3, and 6 after inoculation and each femur was assayed separately. These specific activity values were of the same magnitude as in the preliminary experiment and are shown in Table I.

TABLE I.—*Experiment EMA-I. Myeloperoxidase Activity of Bone Marrow Extracts from Mice with Ehrlich Ascites Carcinoma*

Group	N	Spec. act. ("e"/g.)	Protein (mg./ml.)
Control:			
Day 0	4	97±7·1*	1·39±0·20*
8	4	79±5·5	1·40±0·04
Experimental:			
Day 1	4	94±13·0	1·46±0·05
3	4	107±9·5	1·34±0·06
6	4	130±9·0	1·48±0·04

* Standard error.

Statistically significant differences in specific activity could not be demonstrated between the controls and day 1 and day 3 animals, but the day 6 mice showed an increase over the controls exceeding three standard deviations, or four times the standard error. When the data are plotted, one finds again a linear increase of MPO specific activity amounting to 7·7 per cent per day. Protein levels were the same in control and experimental extracts.

The experiment was repeated with eight control and twelve experimental mice (Expt. EMA-II) and in this case the experimental animals were sacrificed on the first 6 consecutive days after inoculation, two on each day. Controls were sacrificed on days 0, 2, 4, and 6. In addition, the bone marrow extracts from the right and left femur of each mouse were prepared with *n*-butanol, pooled, and stored at — 70° C. for 2 weeks before assay. These results are shown in Table II. Here one observes that both the control and experimental specific activity values are substantially higher than those obtained in the previous experiment. Furthermore, the experimental levels are appreciably higher than the controls, + 58·5 per cent, and this increase appears to have occurred abruptly. This was the only experiment which yielded such high values, and the only one in which the extracts prepared with *n*-butanol were stored before assay. As mentioned above, these results indicate that storage of extracts at — 70° C. can liberate additional activity. However, preliminary tests of this observation gave inconsistent results. One gains the impression that liberation of activity and destruction of enzyme under these conditions are occurring simultaneously and at different rates. A comparison of this experiment with the preceding one suggests that there may be two forms of myeloperoxidase, that one form is readily extractable under the conditions described above, and that the other form becomes progressively more extractable when a neoplastic process is present. The higher values for both the control and experimental extracts of experiment EMA-II are probably due to the presence of *n*-butanol in the extract plus storage at — 70° C. These condi-

TABLE II.—*Experiment EMA-II. Myeloperoxidase Activity of Bone Marrow Extracts from Mice with Ehrlich Ascites Carcinoma*

Group	N	Spec. act. ("e"/g.) (ave.)	Protein (mg./ml.) (ave.)
Control:			
Day 0	2	123	1·55
2	2	188	1·71
4	2	130	2·40
6	2	166	1·61
Mean:		152	1·82
Experimental:			
Day 1	2	263	1·51
2	2	248	1·24
3	2	221	1·67
4	2	212	1·40
5	2	278	1·40
6	2	226	1·48
Mean:		241	1·45

tions appear to bring about the abrupt release of activity which would otherwise occur gradually as the tumor developed. If one assumes that the values for total MPO specific activity in experiment EMA-I should be higher by 64 "*e*"/g. (152–88), the day 6 value would become 194, indicating that as the neoplasia progresses, the liberation of total MPO activity is being approached.

C-1498 *myelogenous leukemia*

In this experiment, four control and twelve experimental mice were employed. The extracts in this instance were prepared without *n*-butanol treatment. These results are presented in Table III. The control values are higher than those observed

TABLE III.—*Myeloperoxidase Activity of Bone Marrow Extracts from Mice with C-1498 Myelogenous Leukemia*

Group	N	Spec. act. ("e"/g.) (ave.)	Protein (mg./ml.) (ave.)
Control:			
Day 1	2	128	1·37
6	2	136	1·57
Experimental:			
Day 1	2	156	1·74
2	2	165	1·50
3	2	177	1·49
4	2	160	1·49
5	2	165	1·39
6	2	130	1·56

in experiment EMA-I; this difference may be characteristic of the C57 Bl mouse. Greenstein (1954, p. 521) has noted that the C57 Bl mouse has a much lower hepatic catalase level than all the other strains studied. The increased MPO

activity is again observed in the tumor-bearing animals; but in this case the level has dropped back to the control value on the sixth day. Again, no significant change in the protein content of the extract was observed.

Sarcoma 180

Nineteen mice were studied in this experiment, the seven control mice receiving sham implants by trocar. The extracts were prepared with *n*-butanol, kept in an ice-bath, and all were assayed within 3 hours after preparation. Extracts of the right and left femur from each mouse were pooled after preparation. In Table IV one will note that the specific activity of the day 0 control mice is higher than the day 2 and day 4 values. This is probably a stress response since the

TABLE IV.—*Myeloperoxidase Activity of Bone Marrow Extracts from Mice with Sarcoma* 180

Group	N	Spec. act. ("e"/g.) (ave.)	Protein (mg./ml.) (ave.)
Control:			
Day 0	2	123	1·66
2	2	92	1·84
4	3	100	1·71
Experimental:			
Day 1	2	124	1·80
2	2	117	1·68
3	2	116	1·73
4	2	123	1·67
5	2	141	1·90
6	2	149	1·78

animals were sacrificed only 4 hours after the sham implant. The higher value for the day 1 experimental mice is also very likely a reflection of the imposed stress. If the average of the day 2 and day 4 controls, 96, is taken as the normal value, we find in the experimental group a 21 per cent increase in MPO activity on day 3, 28 per cent on day 4, 47 per cent on day 7, and 55 per cent on day 9. This response is essentially linear, approximating 5·8 per cent per day. The protein content of the extracts remains unchanged.

DISCUSSION

In the three different types of experimental tumor investigated, an increase in the level of bone marrow myeloperoxidase activity was observed, associated with early neoplasia. The normal C57 Bl mouse, which shows a lower hepatic catalase level than other strains, shows a higher bone marrow MPO level and, in contrast to the Swiss Webster mouse, showed a rise and then a fall in MPO activity in the presence of a growing tumor. With the Ehrlich ascites carcinoma and Sarcoma 180 we have found a progressive increase in bone marrow MPO activity. The results in experiment EMA-II suggest that the presence of tumor cells is not only associated with a higher level of MPO activity in the bone marrow, but may also bring about a progressive liberation of the enzyme or its active centers. This observed increase in MPO does not support the suggestion of Fukuoka

and Nakahara (1951) that the presence of a tumor may result in the binding of iron with a consequent depression of all iron-porphyrin-containing enzymes.

Since very little is known about the physiological function of animal peroxidases one can do little more than speculate on the significance of these findings. Neufeld *et al.* (1955) observed an increase in rat uterine peroxidase in response to estrogen administration and Lucas *et al.* (1955) were able to detect peroxidase activity in the Walker 256 rat carcinoma. They suggested that the enzyme might be involved in terminal respiration. The adaptive response of liver tryptophan peroxidase to elevated tryptophan levels (Knox and Mehler, 1950, 1951; Price and Dietrich, 1957) and of cytochrome-*c* peroxidase of *Pseudomonas fluorescens* to low oxygen tensions (Lenhoff and Kaplan, 1956) suggests that the increase in MPO activity in the presence of tumor may also be a type of adaptive response. Much more work is required to elucidate the true significance of these findings.

SUMMARY

1. Evidence was presented showing a progressive increase in the level of myeloperoxidase activity in bone marrow extracts of mice bearing the Ehrlich ascites carcinoma, C-1498 myelogenous leukemia, and Sarcoma 180. The C57 Bl mouse which normally has a lower hepatic catalase level than the Swiss Webster mouse was found to have a higher bone marrow MPO level. In this strain the bone marrow MPO level of the tumor-bearing mouse dropped back to normal on day 6 after implantation.

2. The results of an experiment with the Ehrlich ascites carcinoma suggested the possibility of two forms of myeloperoxidase with one form being increased in the presence of neoplasia, and progressively liberated as the neoplastic growth develops.

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